

**CERTIFICATION THAT TRANSLATION IS TRUE AND ACCURATE**

I, Cai-xia WANG, state that the English translation  
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王彩霞

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## **A CARDIO MYOPEPTIDIN AND THE USE THEREOF**

### **Abstract**

The present invention relates to a cardio myopeptidin which is isolated from the hearts of non-human healthy mammals. The molecular weight of the cardio myopeptidin is less than 10000 Dalton, the peptide content thereof being from 75% to 90%, the free amino acid content from 6% to 15%, the ribonucleic acid content less than 2%, and the deoxyribonucleic acid content less than 7.5%. The present invention further provides determinations of the biological activity of cardio myopeptidin and the use thereof in producing pharmaceuticals for treating cardiac disorders, specifically the use in producing pharmaceuticals for treating myocardial ischemic and reperfusion injury. The cardio myopeptidin of the present invention can work directly on myocytes, promoting the repair of injuries caused by various reasons, and providing a new way to relieve ischemic and reperfusion injury, and to promote the repair of injured myocardium.

## Claims

1. A cardio myopeptidin, which is a polypeptide isolated from hearts of healthy non-human mammals, comprising 75%~90% of peptide, 60%~15% of free amino acid, less than 2% of ribonucleic acid, less than 7.5% of deoxyribonucleic acid, and molecular weight is less than 10000 Da.
2. The cardio myopeptidin of claim 1 wherein said non-human mammals comprises pigs, cattle, sheep, rabbits or horses.
3. The cardio myopeptidin of claim 2 wherein said non-human mammals comprises infant pigs, infant cattle, infant sheep, infant rabbits or horses, preferably infant pig.
4. The cardio myopeptidin of claim 1 wherein average molecular weight is in the range from 1000 to 10000 Da.
5. The cardio myopeptidin of claim 1 wherein average molecular weight is preferably in the range from 2000 to 8000 Da, and more preferably from 2000 to 5000 Da.
6. The cardio myopeptidin of any one of claim 1 to 5 wherein the biological activity of cardio myopeptidin is stable at pH from 3 to 8, the cardio myopeptidin is sensitive to protease K, the biological activity will not change at the temperature of 85°C for 10 minutes, and is stable under frozen or lyophilized condition.
7. The cardio myopeptidin of any one of claim 1 to 5 wherein isoelectrofocusing electrophoresis of said cardio myopeptidin displays 2~6 stained bands.
8. The cardio myopeptidin of claim 7 wherein isoelectrofocusing electrophoresis of said cardio myopeptidin displays two bands, among which, the band whose pI is 10.92 is the one with deeper color.
9. The cardio myopeptidin of any one of claim 1 to 5 wherein said cardio myopeptidin has a stable maximum absorption peak at 190~210 nm wavelength in UV spectrum.
10. The cardio myopeptidin claim 8 wherein said cardio myopeptidin has a stable maximum absorption peak at  $200 \pm 2$  nm wavelength in UV spectrum.
11. The cardio myopeptidin of any one of claim 1 to 5 wherein the activity of said cardio myopeptidin is 2.2.
- 12 The cardio myopeptidin of any one of claim 1 to 5 wherein said cardio myopeptidin further comprises excipient, and the content by weight is:  
cardio myopeptidin: 15~20  
excipient: 100~375,  
preferably the content is 18~20: 200~375,  
and the excipient is mannitol, trehalose, lactose or sucrose or other adjuvants for lyophilization; preferably mannitol.
13. The cardio myopeptidin of claim 12 wherein said cardio myopeptidin further comprises activity carbon with the content about 0.1%.
14. The cardio myopeptidin of any one of claim 1 to 5 wherein said cardio myopeptidin show five peaks on FPLC analysis spectrum, and the sum of relative area is 90%~95%.
15. The use of cardio myopeptidin of claim 1 for the manufacture of a medicament for the treatment of cardiovascular disease.
16. The use of cardio myopeptidin for the manufacture of a medicament for the treatment of myocardial ischemia-reperfusion injury.

## **Description**

### **TECHNICAL FIELD**

The present invention relates to a myocardial polypeptide and the use thereof. More specifically, this invention relates to a myocardial polypeptide (cardio myopeptidin) isolated from the hearts of healthy mammals other than a human and the use thereof. The present invention belongs to the field of biochemistry.

### **BACKGROUND OF THE INVENTION**

Myocardial protection" has been a hot subject in studies conducted by cardiac medicine and cardiac surgery in recent years. Recent documents showed that, ischemia and hypoxia could cause many changes to the myocardial cells, including overload of calcium in the cells, generation of free radicals, valvular injury, decrease in ATP (adenosine triphosphate, ATP) level, oxygen exhaustion, etc.

The consummation and popularization of cardiac surgical interventions have released numerous patients from pains and improved their life quality. With the increasingly higher requirements of people for myocardial protection, a lot of people have been in fundamental and clinical studies. The myocardial protection in clinical cardiac surgery includes myocardial protection prior, during and after the surgical operation, yet the focus is still on the prevention of ischemia and reperfusion injury in the course of extracorporeal circulation for cardiac arrest. For this purpose, the fundamental and clinical researchers have been aggressively tackling the issues of myocardial protection, which mainly include: (1) the way of perfusion, for example, direct perfusion, inverse perfusion, simultaneous perfusion, intermittent perfusion or continuous perfusion; and whether a blood cell filter is used, and so on; (2) temperature of the perfusate: mainly including normal temperature, low temperature, etc.; (3) components of the perfusate: such as the additional use of the oxygen free-radical scavenging of super-oxide dismutase, reduced glutathione, aprotinin, or puerarin, etc. These measures have, to a certain degree, changed the pathological processes of the myocardial ischemia-reperfusion injury. Some foreign researchers tried to put phosphokinase (Neoton from Italian OUHUI Pharmaceutical Plant) in the perfusate or have patients take trimetazidine dihydrochloride (Vasorel from French Servier), so as to improve the metabolism of cardiac muscle, and elaborated some of their findings from aspects of cell, subcellular structure, oxygen free radicals, energy metabolism, calcium ion ( $\text{Ca}^{2+}$ ) overload and so on. However, these studies either focused only on the supplement of energy or separated the prevention of damage from the defense mechanism of the organism itself, which led to the unsatisfactory clinical outcome in preservation of the cardiac myocardium. Therefore, it is necessary to research and develop more efficacious therapeutic agents for myocardial protection.

In order to interfere with myocardial ischemia and protect heart muscle, many drugs were developed in the past twenty years, such as .beta.-blockers, calcium antagonists, converting enzyme inhibitor, various oxygen free-radical scavengers etc, but their protective effect on cardiac muscle is not affirmed clinically yet. None of the existing medicine for the treatment of myocardial ischemia can absolutely decrease myocardial infarction and antagonize myocardial ischemia. The alteration in the salivary gland chromosome of the fruit fly *Drosophila* following a short-term elevation in the temperature was reported in the late 1980s, indicating genetic transcription was activated, which was called heat shock reaction (HSR, Anna Rev Biochem 1986, 55:1151). Thereafter, it was found that the heat shock preconditioning of experienced animals could obviously decrease damage to the myocardium caused

by ischemia/perfusion, and it was named "ischemia precondition" (IP) by Marry in 1986. Further research indicated that a new group of proteins, that is heat shock proteins (HSPs), or so-called stress proteins (SP), were induced and synthesized by heat shock of cardiac myocytes. These successive research reports demonstrated that heart muscle itself has strong cellular tolerance; later, it was found that HSPs can be induced and expressed under the heat shock treatment for both cultured cells and the whole organism, such as prokaryotes, eukaryotes, plants, animals and human beings. The features of HSR and HSPs is shown as following: (1) Besides induction by heat shock, HSP could be synthesized by many other factors, such as ischemia, hypoxia, ethanol, heavy metallic salt, myocardial pressure load, drugs and most disease, and could produce "cross tolerance phenomenon". (2) HSP had high conservatism in structure; for example, 72% of amino acid sequence of HSP<sub>70</sub> is identical for fruit fly and saccharomycete, 73% of HSP<sub>70</sub> gene is homologous for human and fruit fly, and 78% for HSP<sub>90</sub>. These structural similarities guaranteed the functional sameness (Burdon: Biochem, J. 1986, 240:313). (3) HSP had an optimum time horizon on myocardial protection, alternatively called "window of opportunity," and it would not provide protection if a certain time limit was exceeded (Perdriget: Curr. Surg., 1989, 23). (4) HSP existed in the whole biological universe and was present in various cells of organisms of higher animals. Induction of HSPs not only enhances rehabilitation of myocardial function, but also reinforces rehabilitation of myocardial endothelial function and extends the cardioplegic arrest time. The above described function can be used in donor protection of heart transplantation, salvage of myocardium ischemic, preparation of cardioplegic solution for extracorporeal circulation, etc., and it can break through the restriction of traditional drugs and provide a milestone new method that starts from enhancing the induction of self anti-damage potential of in-vivo cells.

Pennica et al (1995) cloned the cardiotrophin-1 (CT-1) gene from myocardial cells, and expressed it in *Escherichia coli*. It was indicated that CT-1 is a type of cytokine with the function of immunoregulation, induction of proliferation, and anti-injury of myocardial cells caused by hypoxia and high temperature. It naturally exists in the myocardial cells and can inhibit the apoptosis of myocardial cells. Further research showed that CT-1 could induce HSP expression in cultured myocardial cells and in vivo. The effect of induction is concerned with a cell surface polypeptide named gp<sup>130</sup>, and the activated gp<sup>130</sup> reinforces the expression of HSP<sub>70</sub>, HSP<sub>90</sub> and micromolecule substrate of HSP through NF-IL-6/NF-IL-6.β and tyrosine kinase path, thus enhancing the ability of the myocardial cells to tolerate hypoxia and high temperature. Studies also indicated that CT-1 could promote synthesis of structural protein of the myocardial cells, and the increase in the long axis of cells could make contraction stronger. The study of myotrophin of gene recombination is another subject of stimulating factors of the myocardial cells. Parames (1997) demonstrated that myotrophin promotes growth of the myocardial cells and is associated with protein kinase-C. Myotrophin and cardiotrophin may all be cytokines with similar function in the myocardial cells and different paths to activate cellular proliferation, and both show the function of protecting the myocardial cells and promoting proliferation.

Among the available cardiovascular drugs, except for converting enzyme inhibitor which has the function of blocking the generation of growth factors, inhibiting protein synthesis and myocardial hypertrophy, other drugs do not have the function of regulating the growth, differentiation and rehabilitation of cardiac muscles. In recent years, inducing the protection of cardiac muscle cells themselves by pharmacological treatment has been emphasized overseas, such as the research on promoting the myocardial regeneration by transduction gene etc, and the study on cardiotrophin and

myotrophin. Moreover, extracellular signals are used to trigger various transmission mechanisms and to regulate and control the proliferation or reconstitution of myocardial and vascular cells. However, all of this research is at the stage of animal testing or preclinical study.

It is clearly demonstrated from aforesaid studies that, in the situation where protection of cardiac muscle for extracorporeal circulation is not consummated yet, it is of great importance to provide a drug that poses no damage to the organism and can protect cardiac muscle prior, during and after surgical interventions. A new approach to explore the prevention and cure of myocardial ischemia and reperfusion injury is also necessary.

ZL94102798 disclosed a growth-stimulating peptide of the myocardial cells and the process for preparation thereof. The process comprises the steps of: the heart of healthy infant mammals other than a human was crushed with mechanical means, deep frozen at -20°C and heated to 60-100°C after dissolving in water, then deep frozen at -20°C and centrifuged at 3000 rpm after being melted, and finally a polypeptide active substance with molecular weight less than 20000 Da was obtained through negative pressure interception column, sterilization, filling, lyophilization and packing.

ZL94102799 disclosed a growth-stimulating peptide of the myocardial cells (GMGSP) that can stimulate DNA synthesis and protein synthesis of primarily cultured myocardial cells, which was isolated from the heart of healthy infant mammals other than a human mammal, and stabilizes at pH 2-9; the biological activity did not change when GMGSP was heated at 95-100°C for 10 minutes or at 60-70°C for 30 minutes, but biological activity was lost when being placed in proteolytic enzymes at 37°C for two hours; a polymer was formed at 22°C-30.°C. in aqueous solution, but biological activity did not have obvious change; biological activity did not change if GMGSP was lyophilized and sealed with 3%-8% mannitol and stored at room temperature for 1.5 years, or at 4°C for 2 years, or at -20°C for 3 years; HPLC analysis indicated that the aforesaid GMGSP is composed of four components. The relative peaks and retention times of each component were respectively 10.4% (2.88 minutes), 6.4% (3.93 minutes), 36.3% (5.09 minutes) and 7.3% (7.41 minutes), and each component has biological activity. The molecular weights of two bands displayed by SDS-PAGE analysis were respectively 8500 Da and 10800 Da. The average molecular weight displayed by HPLC analysis was 9800 Da, average molecular weight was 10500 Da, and both components have biological activity.

However, the biologically active peptides described in above-mentioned patents are obtained by a rough separation, purification, the test of activity is also simple, and it fails to provide detailed description for its ingredients, use and efficacy.

## **SUMMARY OF THE INVENTION**

The first object of the present invention is to provide a cardio myopeptidin. The major active ingredient of cardio myopeptidin is a polypeptide, which can act on the myocardial cell directly and promote the repair of myocardial damage caused by multiple factors. This invention provides a new approach to decrease myocardial damage in cardiac surgery and promote repair of damage.

The second object of the present invention is to provide the use of cardio myopeptidin for the manufacture of a medicament for the treatment of cardiovascular disease.

The third object of the present invention is to provide the use of cardio myopeptidin for the manufacture of a medicament for the treatment of myocardial ischemia-reperfusion injury.

According to one aspect of the present invention, there is provided a cardio myopeptidin, which is a polypeptide isolated from hearts of healthy non-human mammals. The peptide content thereof is from 75%.to.90%, the free amino acid content is from 6%.to.15%, the ribonucleic acid (RNA) content is less than 2%, the deoxyribonucleic acid (DNA) content is less than 7.5%, and the average molecular weight is less than 10000 Da.

The above-mentioned healthy non-human mammals comprise pigs, cattle, sheep, rabbits, horses and so on. It is preferred the infant mammals are chosen from pigs, cattle, sheep, rabbits, horses etc. Infant pigs are more preferred.

The average molecular weight of cardio myopeptidin is less than 10000 Da, preferably in the range from 1000 to 10000 Da, more preferably in the range from 2000 to 8000 Da, and the most preferably in the range from 2000 to 5000 Da.

The biological activity of cardio myopeptidin is stable at pH from 3 to 8. The cardio myopeptidin is sensitive to protease K. The biological activity will not change at the temperature of 85.°C. for 10 minutes, and is stable under frozen or lyophilized conditions.

Isoelectrofocusing electrophoresis of cardio myopeptidin displays from 2 to 6 stained bands. It is preferred that isoelectrofocusing electrophoresis displays two bands, among which the band whose pI is 10.92 is the one with deeper color.

The cardio myopeptidin of the present invention has a stable maximum absorption peak at from 190 to 210 nm wavelength in the UV spectrum. It is preferred the maximum ultraviolet absorption peak is at 200.±.2 nm wavelength.

Sulfosalicylic acid reagent test indicates that no protein is contained in the cardio myopeptidin of the present invention.

The activity of the cardio myopeptidin of the present invention is at least 2.2.

The cardio myopeptidin of the present invention further comprises excipient, and the content by weight is: cardio myopeptidin: from 15 to 20, excipient: from 100 to 375. Above which, it is preferred the content is from 18 to 20: from 200 to 375.

The excipient may be mannitol, trehalose, lactose, sucrose or other adjuvants for lyophilization, preferably mannitol.

In order to depyrogenate, the cardio myopeptidin may further comprise activated carbon with the content about 0.1%.

The cardio myopeptidin of this invention principally showed five peaks on FPLC analysis spectrum, and the sum of relative area is from 90% to 95%. An activity test indicates that the five peaks can all promote the activity of succinic dehydrogenase of primarily cultured myocardial cells and the myocardial cells with oxygen re-supplied due to lack of oxygen, among which the activity of peak P1 is comparatively high.

The present invention also provides a method for preparing the cardio myopeptidin, which comprises the steps of: (a) cleaning and cutting the hearts of healthy non-human mammals; (b) homogenizing by adding sterile distilled water to the myocardium of healthy non-human mammal which is cleaned and cut; (c) freezing and thawing cycles of the homogenate alternately for 3 or 4 times; (d) filtering by the plate-and-frame filter to get a coarse filtrate and removing the residue after the homogenate is heated to from 65 to 95.°C.; (e) ultra-filtering the coarse filtrate with a hollow-fiber column to get a fine filtrate; (f) ultra-filtering the fine filtrate by ultrafiltration membrane to intercept the cardio myopeptidin solution with the molecular weight less than 10000 Da; (g) concentrating the solution by reverse



osmosis to get a concentrated cardio myopeptidin solution. The method may further comprise the steps of testing the quality, filtering aseptically, filling and lyophilizing.

The amount of sterile distilled water added is from 0.5 to 4 times of that of the myocardium of mammals, and the rotation speed of homogenization is in the range from 1000 to 5000 rpm/min.

The freezing is performed at a temperature of less than  $-5^{\circ}\text{C}$  for from 24 to 72 hours, preferably at from  $-20^{\circ}\text{C}$  to  $-30^{\circ}\text{C}$  for from 36 to 48 hours; heating is in the way of water bath heating or direct heating at a temperature of from  $70^{\circ}\text{C}$  to  $90^{\circ}\text{C}$  for not more than 2 hours, and preferably water bath heating at a temperature of from  $75^{\circ}\text{C}$  to  $80^{\circ}\text{C}$  for 1 hour.

In the present invention, the coarse filtrate is obtained through a plate-and-frame filter, fine filtrate with molecular weight less than 12 k Da is obtained through a hollow fiber column, and final filtrate with molecular weight less than 10 k Da is obtained by intercepting part of solution through ultrafiltration membrane. The plate-and-frame filter is conventional biopharmaceutical equipment with the medium-speed filter paper having pores less than  $10\ \mu$ , preferably the pores less than or equal to  $5\ \mu$ , such as the XAS03-172/8 model plate-and-frame filter manufactured by Guangzhou Medicinal Apparatus Research Institute. F60 model hollow fiber column which can filter liquid with a molecular weight less than 12 k Da is introduced, such as hollow fiber columns produced by Sweden Gambro. The specification of the ultrafiltration membrane is from 1 to 10 k Da, such as the products of Millipore Corporation, and the reverse osmosis/concentration column is also the product of Millipore Corporation.

The aseptic filtration and filling processes described in the present invention are prior art and are well known by a person skilled in the art.

The current lyophilizer is introduced for freeze-drying the cardio myopeptidin in the present invention. The process of lyophilization comprises the steps of: the shelf in the drying chamber is cooled down to the temperature of from  $-15^{\circ}\text{C}$  to  $-20^{\circ}\text{C}$  in from 5 to 40 minutes, preferably to from  $-18^{\circ}\text{C}$  to  $-20^{\circ}\text{C}$  in from 20 to 30 minutes, followed by the cardio myopeptidin being frozen to the temperature of from  $-25^{\circ}\text{C}$  to  $-35^{\circ}\text{C}$  within from 20 to 40 minutes and maintaining at this temperature for from 1 to 3 hours, preferably to from  $-30^{\circ}\text{C}$  to  $-35^{\circ}\text{C}$  within from 25 to 35 minutes; then the condenser is chilled to the temperature of from  $-40^{\circ}\text{C}$  to  $-50^{\circ}\text{C}$ . At that time, the pressure is reduced till the vacuum degree reaches from 90 to 100 Kpa, the drying chamber is connected with condenser, and the refrigeration is stopped. After that, the temperature of drying chamber is raised to from  $5^{\circ}\text{C}$  to  $15^{\circ}\text{C}$  at the rate of from  $2^{\circ}\text{C}$  to  $5^{\circ}\text{C}/\text{min}$  and maintained at this temperature for from 3 to 6 hours when the vacuum degree of the drying chamber gets to from 10 to 15 Pa, preferably the temperature is ascended to from  $8^{\circ}\text{C}$  to  $12^{\circ}\text{C}$  at the rate of from  $3^{\circ}\text{C}$  to  $4^{\circ}\text{C}/\text{min}$  with from 4 to 5 h heat preservation. The temperature is elevated continuously to from  $15^{\circ}\text{C}$  to  $25^{\circ}\text{C}$  at the rate of from  $8^{\circ}\text{C}$  to  $16^{\circ}\text{C}/\text{min}$  and kept for from 3 to 8 hours, preferably the temperature is raised to from  $18^{\circ}\text{C}$  to  $22^{\circ}\text{C}$  at the rate of from  $10^{\circ}\text{C}$  to  $12^{\circ}\text{C}/\text{min}$  for from 4 to 6 hours. Then the temperature is further increased continuously to from  $30^{\circ}\text{C}$  to  $35^{\circ}\text{C}$  at the rate of from  $7^{\circ}\text{C}$  to  $15^{\circ}\text{C}/\text{min}$  and maintained for from 1 to 4 hours, preferably from  $33^{\circ}\text{C}$  to  $35^{\circ}\text{C}$  at the rate of from  $9^{\circ}\text{C}$  to  $12^{\circ}\text{C}/\text{min}$  for from 1.5 to 2 hours. Furthermore, the temperature is raised continuously to from  $50^{\circ}\text{C}$  to  $60^{\circ}\text{C}$  at the rate of from  $4^{\circ}\text{C}$  to  $8^{\circ}\text{C}/\text{min}$  lasting for from 1 to 3 hours, preferably to from  $54^{\circ}\text{C}$  to  $58^{\circ}\text{C}$  at the rate of from 5

to 7.°C/min for from 1.5 to 2 hours. Then comes the cooling stage, in which the temperature is cooled down to from 40 to 50°C within from 10 to 30 minutes and stands at such temperature for from 8 to 15 hours, preferably cooled down to from 45 to 48 °C in from 15 to 20 minutes and from 9 to 12 h preservation at such temperature to attain lyophilized production of cardio myopeptidin with qualified appearance.

In the process of preparing the cardio myopeptidin of this invention, a known adjuvant for lyophilized product may be introduced to the cardio myopeptidin solution, such as mannitol, trehalose, lactose, sucrose or other adjuvant as the auxiliary for lyophilization; the addition of adjuvant makes it easy to form a crystal lattice, which works as a bracket and stabilizes the product.

The cardio myopeptidin with the molecular weight less than 10000 Da can be obtained by filtering with a plate-and-frame filter, ultrafiltration with a hollow fiber column and ultrafiltration membrane respectively, and concentrating with reverse osmosis according to the process of this invention. Compared with the process described in ZL94102798 in the background, the process of the present invention enables a short operating time to obtain a large quantity of products with high concentration and activity but without pyrogen.

Furthermore, the present invention provides the use of cardio myopeptidin for the manufacture of a medicament for the treatment of cardiovascular disease.

Moreover, the present invention provides the use of cardio myopeptidin for the manufacture of a medicament for the treatment of myocardial ischemia-reperfusion injury.

Compared with the growth-stimulating peptide of the myocardial cells (GMGSP) disclosed in Chinese patents ZL94102798 and ZL94102799, cardio myopeptidin of the present invention has obviously higher in vitro biological activity. The biological activity of cardio myopeptidin of the present invention is from 3 to 5 times higher than that of the growth-stimulating peptide of the myocardial cells. Comparison data of in vivo drug efficacy shows that cardio myopeptidin poses a favorable impact on the release of myocardial creatine phosphokinase caused by myocardial ischemia-reperfusion injury, activity of lactate dehydrogenase, and contents of free fatty acid and malondialdehyde (MDA).

Cardio myopeptidin of this invention can directly act on myocardial cells and promote the repair of myocardial damage caused by multiple damage factors (such as ischemia, drug intoxication etc), and is a drug for promoting protein synthesis, reducing damage of oxygen free radicals, decreasing overload of calcium, inducing endogenous protection and improving the myocardial metabolism. The present invention provides a new approach to lessen the myocardial damage in cardiac surgical operations and promote the repair of injury.

Main pharmacodynamic and pharmacological findings of cardio myopeptidin of this invention are as follows:

1. Cardio myopeptidin can obviously lessen the damage of the myocardial ultrastructure caused by myocardial ischemia-reperfusion and make it approach or return to normal condition (FIG. 6-12 black-and-white photo and Table 13).

2. It is demonstrated by the electrocardiogram of epicardium that cardio myopeptidin can obviously antagonize ST elevation caused by myocardial ischemia in cats and reduce the scope of myocardial ischemia (Table 14-15).

3. Cardio myopeptidin can obviously lower the release of myocardial creatine phosphokinase caused by myocardial ischemia-reperfusion injury, increase the activity of lactate dehydrogenase, and enhance the contents of free fatty acid and

malondialdehyde (Table 16-21).

4. The oxygen consumption of cardiac muscles can be reduced by cardio myopeptidin (Table 22).

5. ST and/or NST in the electrocardiogram of pigs with myocardial infarction can be significantly reduced or decreased by administering cardio myopeptidin at a dose of 5 mg/kg or 10 mg/kg per body weight, and the scope of myocardial infarction can also be reduced. Cardio myopeptidin has certain therapeutic effects on arrhythmia and ventricular fibrillation (they may cause death) in pigs with acute myocardial ischemia, but has no evident impact on blood pressure and heart rate (Table 23 and FIG. 13).

## **BRIEF DESCRIPTION OF THE DRAWINGS**

To understand the present invention, it will now be described by way of example, with reference to the accompanying drawings in which:

FIG. 1 is an HPLC spectrum of molecular weight of cardio myopeptidin of the present invention;

FIG. 2 is a FPLC spectrum of separating and purifying cardio myopeptidin of the present invention;

FIG. 3 is an isoelectrofocusing electrophoresis spectrum of cardio myopeptidin of the present invention;

FIG. 4 is a UV spectrum of cardio myopeptidin of the present invention;

FIG. 5 is an HPLC spectrum for identification of the cardio myopeptidin for injection of the present invention;

FIG. 6 is a photograph of electron microscope for pseudo-operation control group in the experiment of influence of cardio myopeptidin on damage of myocardial ultrastructure caused by ischemia-reperfusion of the present invention;

FIG. 7 is a photograph of electron microscope for normal saline reference group in the experiment of influence of cardio myopeptidin on damage of myocardial ultrastructure caused by ischemia-reperfusion of the present invention;

FIG. 8 is a photograph of electron microscope for ischemia-reperfusion reference group in the experiment of influence of cardio myopeptidin on damage of myocardial ultrastructure caused by ischemia-reperfusion of the present invention;

FIG. 9 is a photograph of electron microscope for 10.0 mg/Kg cardio myopeptidin group in the experiment of influence of cardio myopeptidin on damage of myocardial ultrastructure caused by ischemia-reperfusion of the present invention;

FIG. 10 is a photograph of electron microscope of FIG. 9 for 5.0 mg/kg cardio myopeptidin group;

FIG. 11 is a photograph of electron microscope of FIG. 9 for 1.0 mg/kg cardio myopeptidin group;

FIG. 12 is a photograph of electron microscope of FIG. 9 for 2.0 mg/kg propranolol group;

FIG. 13 is a diagram illustrating ST and/or NST in the electrocardiogram of pigs with myocardial infarction being significantly reduced by administered cardio myopeptidin at a dose of 5 mg/kg or 10 mg/kg of body weight.

## **DETAILED DESCRIPTION**

The following preferred embodiments further describe this invention, and said preferred embodiments are only used to describe instead of limit this invention.

### **EMBODIMENT 1**

This experiment relates to the physicochemical property, purity, content and activity test of cardio myopeptidin solution.

1. Physicochemical property, purity and content of cardio myopeptidin solution.

Cardio myopeptidin of this invention is a small molecular active polypeptide, and its biological activity is stable at pH from 3 to 8 and is not changed at 85°C for 10 minutes. Cardio myopeptidin is sensitive to protease K and is stable under frozen or lyophilized condition. The average molecular weight is less than 10000 Da analyzed by HPLC spectrum (shown in FIG. 1), preferably between 2000-8000 Da.

TABLE 1 Influence on activity of cardio myopeptidin at different pH and different time period (MTT method) ( $\bar{x} \pm s$ ,  $n = 8$ )

		Activity of cardio myopeptidin OD value ( $\bar{x} \pm s$ )	
Group		30 $\mu$ g/ml	5 $\mu$ g/ml
Normal control group		0.691 $\pm$ 0.032**	
Adriamycin group		0.274 $\pm$ 0.011	
Different pH group			
3.0	30min	0.331 $\pm$ 0.014**	0.320 $\pm$ 0.015**
	60 min	0.314 $\pm$ 0.007**	0.309 $\pm$ 0.010**
4.0	30 min	0.315 $\pm$ 0.008**	0.307 $\pm$ 0.015**
	60 min	0.334 $\pm$ 0.015**	0.311 $\pm$ 0.007**
5.0	30 min	0.364 $\pm$ 0.022**	0.379 $\pm$ 0.019**
	60 min	0.364 $\pm$ 0.017**	0.353 $\pm$ 0.023**
6.0	30 min	0.341 $\pm$ 0.023**	0.344 $\pm$ 0.011**
	60 min	0.332 $\pm$ 0.015**	0.327 $\pm$ 0.016**
7.0	30 min	0.320 $\pm$ 0.018**	0.358 $\pm$ 0.023**
	60 min	0.327 $\pm$ 0.010**	0.328 $\pm$ 0.012**
8.0	30 min	0.339 $\pm$ 0.008**	0.332 $\pm$ 0.022**
	60 min	0.308 $\pm$ 0.01**5	0.309 $\pm$ 0.010**
9.0	30 min	0.313 $\pm$ 0.006**	0.289 $\pm$ 0.020
	60 min	0.279 $\pm$ 0.017	0.274 $\pm$ 0.013

Note: \*\*Comparing with Damage Group  $P < 0.01$

It can be seen from Table 1 that the biological activity of cardio myopeptidin is stable at pH from 3 to 8.

The cardio myopeptidin of this invention principally showed five peaks on FPLC analysis, and the sum of relative area is from 90% to 95%. It is indicated by activity test that the five peaks can all promote the activity of succinic dehydrogenase of primarily cultured myocardial cells and the myocardial cells with oxygen re-supplied due to lack of oxygen (Table 2), among which the activity of peak P1 is comparatively high. Polypeptide content in cardio myopeptidin is 75-90.quadrature., free amino acids content is 6-15.quadrature., and there is a little nucleic acid and microelement. Isoelectrofocusing electrophoresis of cardio myopeptidin displays two stained bands, among which the band of pI 10.92 is the one with deeper color (shown in FIG. 3).

TABLE 2 Influence of each peak of cardio myopeptidin on enzyme activity of myocardial cells (MTT method) ( $n = 8$ ,  $\bar{x} \pm s$ )

OD value ( $\bar{x} \pm s$ )		
Group	5 $\mu$ g/ml	t

Normal control group	0.344±0.014**	9.93
Adriamycin group	0.272±0.015	
P1	0.318±0.004**	6.344
P2	0.295±0.012**	3.39
P3	0.309±0.012**	5.45
P4	0.317±0.017**	5.61
P5	0.303±0.014**	4.27
Cardio myopeptidin	0.298±0.005**	3.47

Note: Compared with Adriamycin group, \*\*P < 0.01

It can be seen from Table 2 that all five component peaks can promote the activity of succinic dehydrogenase of primarily cultured myocardial cells.

#### 1.1. Identification of Polypeptide

1 ml of cardio myopeptidin solution with the concentration of 2.5 mg/ml is dissolved with 2 ml of water, in which 2 ml of biuret reagent is added and mixed well. [Preparation of biuret reagent: 0.75 g of copper sulphate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) and 3 g of potassium sodium tartrate ( $\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$ ) is dissolved with 250 ml of water, to which 150 ml of 10% sodium hydroxide is added while stirring and diluted with water to 500 ml, then store the solution in a plastic bottle.] If the solution contains polypeptide, a royal purple solution will be produced. After testing samples from 6 batches, cardio myopeptidin of this invention showed royal purple, which indicates that cardio myopeptidin of this invention contains polypeptide.

#### 1.2 Assay

##### (1) Semi-Micro Kjeldahl Method for the Determination of Nitrogen

Test sample: cardio myopeptidin solution & cardio myopeptidin for injection. Cardio myopeptidin solution with the Batch No. 960419, 960422 and 960423; cardio myopeptidin for injection with the Batch No. 960501, 960502 and 960503 is dissolved with water to required concentration before testing.

Reagents: sulfuric acid: chemically pure and specific gravity is 1.84; digestion reagent: mixture of 1 unit of copper sulphate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) and 10 units of potassium sulphate ( $\text{K}_2\text{SO}_4$ ) ground into fine particles; 12.5 mol/L of sodium hydroxide solution; 2% boric acid absorption solution; 10% sodium tungstate; 0.33 mmol/L of sulfuric acid; mixed indicator: mixture of 5 units of 0.2% (w/v) bromcresol green alcoholic solution and 2 units of 0.1% (w/v) methyl red alcoholic solution; and 0.01 mol/L hydrochloric acid.

Calculation formula: 
$$\frac{\text{Total nitrogen content in test sample product (g/L)} = \text{Titration volume of sample-titration of blank} \times \text{conc of standardized hydrochloric acid}}{\text{Volume of sample (ml)}}$$

Determination: Proceed as directed under Determination of Nitrogen (see Method 2, Appendix VII D, Volume II of CHINA PHARMACOPOEIA, refer to CP hereinafter, 1995).

Inorganic nitrogen: 5 ml of sample is measured accurately, to which 3 ml of water, 1 ml of 10% sodium tungstate, and 0.33 mmol/L sulfuric acid are added and mixed well. The mixture solution is filtered after standing for 30 minutes. 5 ml of the filtrate and 5 ml of sodium hydroxide test solution are transferred accurately to a distillation flask, and the test proceeds as directed under the above-mentioned Determination of Total Nitrogen.

Total nitrogen: a vial of cardio myopeptidin for injection is dissolved in 4 ml of water, then 2.0 ml of the injection solution is measured accurately, while 2.0 ml of

cardio myopeptidin solution is measured accurately, and the test proceeds separately as directed under the above-mentioned Determination of Total Nitrogen.

Organic nitrogen=Total nitrogen-inorganic nitrogen.

Note: (1) During determination of inorganic nitrogen, because foam may easily be produced in the distillation process, which will lead sodium hydroxide being taken into the condenser tube and subsequently flow into the collected liquid, the determination result will be an upper bound to the true value. Therefore, 10% sodium tungstate and 0.33 mmol/L sulfuric acid are added before distillation to remove organic substances, then inorganic nitrogen is determined by the filtrate.

(2) The main components of the test sample (cardio myopeptidin) are polypeptide substances. Inorganic compound used in the manufacturing process may produce inorganic nitrogen and affect the determination result. After determining the content of total nitrogen by this method, the content of inorganic nitrogen in the test sample is determined, and the difference of the total nitrogen content minus inorganic nitrogen content gives the organic nitrogen content of the present invention.

#### Results & Analysis

Nitrogen content of cardio myopeptidin solution and cardio myopeptidin for injection with different batch numbers is shown in Table.

TABLE 3 Measurement results of nitrogen content in test samples with different batch numbers

	Cardio myopeptidin solution (mgN/ml)			Cardio myopeptidin for injection (mgN/vial)		
Batch No	960419	960422	960423	960501	960502	960503
Total N	1.788	1.926	1.628	3.816	4.250	4.100
Organic	1.589	1.743	1.460	3.612	3.919	3.722
Inorganic	0.199	0.183	0.168	0.204	0.331	0.378

Table 3 shows that the organic nitrogen content of cardio myopeptidin solution is 1.46-1.74 mg/ml, and that of cardio myopeptidin for injection is 3.61-3.92 mg/vial, and the average content is 1.60 mg nitrogen/ml and 3.75 mg nitrogen/vial, respectively.

#### (2) Folin-Phenol Reagent Method

Cardio myopeptidin solution with the Batch No. of 960419, 960422 and 960423; Cardio myopeptidin for injection (CMI) with the batch No of 960501, 960502 and 960503, dissolved with water to proper concentrations before measurement; Reference substance: Bovine serum albumin with Batch No. 9607 (provided by the National Institute for the Control of Pharmaceutical and Biological Products).

Apparatus: Model 7221 spectrophotometer, Shanghai.

Preparation of reagents:

4% sodium carbonate solution: 4 g of sodium carbonate ( $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$ ) in 100 ml of water.

0.2 mol/L sodium hydroxide solution: 0.8 g of sodium hydroxide (NaOH) in 100 ml of water.

1% copper sulphate solution: 1 g of copper sulphate ( $(\text{CuSO}_4 \cdot 5\text{H}_2\text{O})$ ) in 100 ml of water.

2% potassium tartrate solution: 2 g of potassium tartrate ( $(\text{K}_2\text{C}_4\text{H}_4\text{O}_6 \cdot 1/2\text{H}_2\text{O})$ ) in 100 ml of water.

Alkaline copper test solution: Take 25 ml each of test solution 1 and 2, and 0.5 ml each of test solution 3 and 4, then mix well.

Phenol Reagent: 100 mg of sodium tungstate ( $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ ) and 25 g of sodium molybdate ( $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ) are put into a 1500 ml flask, to which 700 ml of water, 50 ml of 85% phosphoric acid and 100 ml of hydrochloric acid are added. A return tube (with cork plug or rubber stopper covered with tin foil) is connected at the top of the flask for boiling and the solution is refluxed in the flask for 10 minutes. Then, the condenser tube is taken off, 150 g of lithium sulfate ( $\text{Li}_2\text{SO}_4$ ), 50 ml of water and several drops of bromine solution are added into the flask and mixed well. The solution is boiled for 15 minutes in the ventilation hood to remove excess bromine. The solution is cooled down to room temperature, and diluted by the water to 1000 ml, and then filtered to obtain the filtrate, which is stored in a brown bottle in a refrigerator, called stock solution. Dilute the stock solution with water before measurement.

#### Plotting of Standard Curve

Preparation of the reference solution: Add 100 mg (accurately weighed) of dried bovine serum albumin reference substance (provided by the National Institute for the Control of Pharmaceutical and Biological Products) into a 100 ml volumetric flask, to which water is added to dilute to scale, and mix well. Add accurately 10.0 ml of the dilution to another 10 ml volumetric flask, and dilute with water to scale and mix well.

Preparation for standard curve: 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of reference solution is added accurately into each of 6 test tubes with stopper and further diluted to 1.0 ml by adding water into each test tube. Then add 5.0 ml of alkaline copper solution to each test tube and mix well, and place the test tube at room temperature for 10 minutes. Then rapidly add 0.5 ml phenol reagent to each test tube and mix well immediately. Stand the test tube in a water bath (35.°C.) for 30 minutes. Remove and cool the test tube to room temperature. Take the test tube with 0.0 ml of reference solution as blank, and determine the absorbance at 660 nm wavelength as directed under spectrophotography (Appendix IV A, Volume II, of CHINA PHARMACOPOEIA 1995). Plot a standard curve by using the absorbance as ordinate and the protein concentration as horizontal coordinate (absorbance vs mg protein).

Determination method: Dissolve and dilute a certain amount of test sample of cardio myopeptidin with water, and add an accurately measured volume of the solution or the dilution to a 50 ml volumetric flask, add water to scale and mix well. Then accurately pipette 10.0 ml mixture to a 50 ml volumetric flask, add water to scale and mix well, and exactly pipette 1.0 ml to a test tube with stopper. After that, proceed with the measurement procedure according to above-mentioned "Preparation for Standard Curve" from `to add alkaline copper solution . . . ` until the determination of absorbance as directed. Check and obtain corresponding concentration from the standard curve, and then calculate.

#### Result and Analysis

The determination result is shown in Table 4.

It is shown from the determination result that the polypeptide concentration of cardio myopeptidin solution is 2.6-2.8 mg per ml, and the content of cardio myopeptidin for injection is 9.2-9.9 mg per vial. In order to keep the content of polypeptide in the solution and in the injection vial constant, we specify that the concentration of polypeptide in cardio myopeptidin solution is more than 2.5 mg/ml, and that in cardio myopeptidin for injection, it is 9.0-11.0 mg/vial.

TABLE 4 Comparison among the determination results of three peptide

# determination methods

	Peptide determination methods		
	Biuret reagent	Folin-phenol reagent	Semi-micro Kjeldahl
CMS** ( mg/ml)			
960419	1.65	2.7	1.58
960422	1.85	2.8	1.74
960423	2.00	2.6	1.46
CMI**(mg/vial)			
960501	3.26	9.9	3.61
960502	3.63	9.2	3.92
960503	3.73	9.2	3.72

Note: \*Biuret reagent method is determined with a fully automatic biochemistry analyzer.

\*\*CMS: cardio myopeptidin solution; CMI: cardio myopeptidin for injection

It can be seen from Table 4 that three peptide determination methods lead to inconsistent results. The reaction principle of the Folin-phenol reagent method lies in the reactivity of phenolic group of aromatic amino acids, which have good specificity and is easy to be manipulate. The use of specific reference substance and the plotting of standard curve in each determination can overcome nonlinear relationship. Thus, Folin-phenol reagent method is taken to determine the content of polypeptide in cardio myopeptidin solution and cardio myopeptidin for injection.

## (3) Analysis of Composition of Cardio Myopeptidin

The cardio myopeptidin of the present invention mainly comprises polypeptides; the organic nitrogen contents of polypeptides and free amino acids are determined separately. The organic nitrogen content of polypeptide is the difference when the organic nitrogen content of free amino acid is subtracted from the total organic nitrogen content; that is, the organic nitrogen content of polypeptide=total organic nitrogen content-the organic nitrogen content of free amino acid.

Reagents and methods are the same as described above (please read the method for the determination of nitrogen).

Result: Table 5 shows the nitrogen content of free amino acids in three batches of cardio myopeptidin for injection.

TABLE 5 Nitrogen content of free amino acids in cardio myopeptidin for injection  
Cardio myopeptidin for injection

Batch No.	960501	960502	960503
Organic nitrogen content of free amino acid g/L	0.257	0.285	0.286

The comparisons between organic nitrogen content of cardio myopeptidin for injection and nitrogen content of free amino acids are shown in Table 6.

TABLE 6 Comparison between the total nitrogen content and nitrogen content in free amino acid

Cardio myopeptidin for injection			
Batch No.	960501	960502	960503
Organic nitrogen content of polypeptides g/L	3.612	3.919	3.722
Organic nitrogen content of free amino acid g/L	0.257	0.285	0.286
Nitrogen content of free amino acid/total organic nitrogen content (%)	6.643	6.823	7.135

From Tables 5 and 6, we can see that the nitrogen content of free amino acids in



cardio myopeptidin for injection accounts for 6.643%-7.135% of the nitrogen content of the test sample, which shows that polypeptide in the test sample accounts for the majority of the nitrogen content. Considering that the polypeptide of this invention is the major component with biological activity and to make the manufacturing process to be stable and controllable, we specify that the percent composition of polypeptide in cardio myopeptidin for injection is in the range from 75% to 90%.

### 1.3 Analysis of Ultraviolet Scanning

Use a Model 2201 ultraviolet spectrophotometer produced by Japanese Shimadzu, and proceed as directed under Spectrophotography (Appendix IV A, Volume II of CHINA PHARMACOPOEIA 1995).

The result shows that the maximum absorption peak of cardio myopeptidin solution is at 199.8-201.2 nm, and the maximum peak of cardio myopeptidin for injection is at 200.4-201.8 nm (FIG. 4), which indicates that the ultraviolet spectra of the three batches of solution and three batches of injection are consistent, the major component of the sample is polypeptide, and the manufacturing process of cardio myopeptidin is stable.

TABLE 7 Ultraviolet absorption wavelength of cardio myopeptidin

Batch No.	Absorption wavelength (nm)
Cardio myopeptidin solution (mg/ml)	
960422	200.4
960423	199.8
960419	201.2
Cardio myopeptidin for injection (mg/vial)	
960501	200.4
960502	201.8
960503	201.6

### 1.4 Identification of Protein

The test solution does not become turbid when 1 ml of 20% sulfosalicylic acid solution is added to 2 ml of cardio myopeptidin solution with the concentration of 2.5 mg/ml of cardio myopeptidin of this invention, and the determination results of three batches of test sample of cardio myopeptidin for solution and injection indicate that no protein is contained therein. Determining protein by the sulfosalicylic-acid test can not only monitor protein absence in the test sample mixed in, but also can demonstrate that the royal purple displayed by the biuret reagent is polypeptide, not any other substance.

### 1.5 Molecular Weight and Detection of Peptide Chromatogram

Molecular weight is determined by the HPLC method with an HP1050 liquid chromatograph.

Chromatographic conditions: mobile phase:sodium sulfate (0.1 mol/L)-sodium dihydrogen phosphate (0.05 mol/L)-sodium azide (0.05%), adjust pH to 6.8 by NaOH; flow rate: 0.35 ml/min; column: TOSOH TSK G2000 sw 7.5 mm.times.300 mm; column temperature: 25.°C.; detection wavelength: 280 nm; sample size: 10 ul.

Reference solutions: add mobile phase to a suitable amount each of cytochrome C (MW=12400), aprotinin (MW=6700) and Vitamin B.sub.12 (MW=1355) respectively to prepare reference solutions with proper concentrations. Test sample solution: add mobile phase to a bottle of cardio myopeptidin for injection that contains 10 mg of polypeptide to get a test sample solution with the concentration of 5 mg/ ml. Inject the reference solution and the test sample solution into the chromatograph according to the chromatographic conditions, and determine the retention time of each solution. The regression equation of the reference substance is obtained by the least square

method with the correlation coefficient not less than 0.99. Plot the standard curve and calculate the molecular weight of the sample from the following formula.

$$\text{IgMW} = A + BtR$$

$$\text{IgMW} = 6.8405 - 0.1219tR \quad r = -0.9990$$

Where MW is the molecular weight, A is a constant, B is the slope, and tR is the retention time (minutes).

Tables 5 and 6 show the results.

TABLE 8 Retention time of the relative area percentage of chromatographic peaks of cardio myopeptidin for injection

Batch No.	Retention time of Chromatographic peak (min)				
	P1	P2	P3	P4	P5
960501	30.5	31.8	32.8	35.0	38.7
960502	30.5	31.2	32.8	35.0	38.7
960503	31.2		32.7	35.0	38.7
X	30.7	31.5	32.7	35.0	38.7

TABLE 9 Molecular weight at peak position of cardio myopeptidin for injection

Batch No.	MW (Da)				
	P1	P2	P3	P4	P5
960501	6023	4588	3665	2233	969
960502	6027	5261	3688	2234	971
960503	5165	3709		2236	875
X	5736	4519	3676	2234	971

$$X = 6.7444 - 0.09696 \times Y = -0.9937$$

The molecular weight of cardio myopeptidin for injection ranges from 922 to 6027 Da. The maximum molecular weight ranges from 5214 to 6027 Da (as shown in FIG. 1). That means the test sample is a polypeptide with a small molecular weight, and its molecular weight is less than 10000 Da. Therefore anaphylactic response will seldom occur when being injected or taken.

#### 1.6 Nucleic Acid

4 ml of distilled water is added to dissolve a bottle of cardio myopeptidin for injection that contains 10 mg of polypeptide. An equal volume of 10 mol/L phenol is added for extracting nucleic acid, then the content of DNA is determined in the supernate. Add two volumes of cold absolute ethyl alcohol and 1/20 volume of 10 mol/L ammonium acetate to the supernate and put it at -80°C for 30 minutes. Centrifuge at 12000 rpm.times.20 min, then discard the supernate, and dissolve the precipitate in 4 ml of distilled water. The dissolved precipitate is the sample to determine the RNA content.

#### (1) Determination of RNA Content

Preparation for standard curve: take 6 test tubes and add in reagents according to following table

Added substance	Added amount (ml)					
Test tube No.	0	1	2	3	4	5
RNA standard solution	0	0.2	0.4	0.6	0.8	1.0
Distilled water	1.0	0.8	0.6	0.4	0.2	0
Orcinol reagent	3.0	3.0	3.0	3.0	3.0	3.0

Mix each tube well and heat in a boiling water bath for 20 min., then take out and

cool down to room temperature with cold water. The absorbance of each tube is determined by a spectrophotometer at 670 nm wavelength, adjusting the zero position of absorbance with the No. "0" tube. Plot a standard curve by using RNA content as the abscissa, and the absorbance as the ordinate.

#### Determination of RNA Content in the Test Sample:

Mark 4 test tubes respectively with "blank tube" and "sample tube." Add 1.0 ml of distilled water to the blank tubes, and 1.0 ml of RNA sample solution to the sample tubes. Add 3.0 ml orcinol reagent in each tube and mix well, then put them in a boiling water bath for 20 minutes. Take out the tubes and cool down to room temperature in a cold bath. The absorbance of each test tube is determined in a spectrophotometer at 670 nm wavelength, adjusting the zero position of absorbance with the blank tube. Finally, RNA content can be obtained by comparing with the standard curve and taking the average value.

#### (2) Determination of DNA Content

Preparation for standard curve: take 6 test tubes and add in reagents according to the following Table.

Added substance	Added amount (ml)					
Test tube No.	0	1	2	3	4	5
RNA standard solution	0	0.2	0.4	0.6	0.8	1.0
DH <sub>2</sub> O	1.0	0.8	0.6	0.4	0.2	0
Diphenylamine reagent	3.0	3.0	3.0	3.0	3.0	3.0

Mix each tube and heat in a water bath at 60.°C. for 60 min, then take out and cool down to room temperature in cold water. The absorbance of each tube is determined by a spectrophotometer at 595 nm wavelength, adjusting the zero position of absorbance with the No. "0" tube. Plot a standard curve by using DNA content as the abscissa, and the absorbance as the ordinate.

#### Determination of DNA Content in the Test Sample:

Mark 3 test tubes respectively with "blank tube" and "sample tube." Add 1.0 ml of distilled water to the blank tube, and 1.0 ml of DNA sample solution in sample tubes, then add 3.0 ml diphenylamine reagent in each tube and mix well. Put into a water bath at 60.°C. for 60 minutes, then take out the tubes and cool down to room temperature in a cold bath. The absorbance of each tube is determined in a spectrophotometer at 595 nm wavelength, adjusting the zero position of absorbance with the blank tube. DNA content can be obtained by comparing with the standard curve and taking the average value.

The results shows that each bottle of cardio myopeptidin for injection contains less than 200 .mu.g (2%) RNA, and the DNA content does not exceed 750 .mu.g (7.5%).

#### 1.7 Activity

Test sample: cardio myopeptidin for injection respectively with the batch number 960501, 960502, 960503, and 960101, and polypeptide content is 10 mg/vial.

Method of primary myocardial cell culture is taken to determine the activity of the present invention.

Experimental results are shown in Table 10.

TABLE 10 t value of activity of cardio myopeptidin for injection (n = 6)

Batch No.	t value
960501	5.8
960502	3.2
960503	7.9
960101	7.8

### 1.8 Identification of Cardio Myopeptidin for Injection by HPLC Method

Apparatus: HP1100, module liquid chromatograph No DE 70300954;

Chromatographic condition: Mobile phase:methanol:water=10:90;

Column: ymc-park ODS-A A-302 150 mm.times.4.6 mm.quadrature.I.D S-5 .mu.m 120A No 041543847 (W);

Column temperature: 26.℃., detecting wavelength: 254 nm, flow rate: 0.8 ml/min, and sample size: 10 .mu.l.

Determination procedure: Add 10 ml of mobile phase to each bottle of the test sample, and the completely dissolved solution is used for the test.

Batch numbers of test sample of cardio myopeptidin for injection are 960101, 960501, 960502, 960503, 961101, 961103, 971201 and 980301, respectively.

The result shows that the 10 batches of test samples mainly display from 4 to 5 principal peaks, and the relative peak area is more than 85%. The retention time of each principal peak is similar (shown in FIG. 5 and Tables 11 and 12).

TABLE 11 Retention time of various principal peaks

Retention (min)									
Peak	101	501	502	503	1101	1102	1103	201	301
P1	1.922	1.901	1.920	1.915	1.925	1.924	1.949	1.954	1.990
P2	2.506	2.504	2.500	2.502	2.643	2.639	2.640	2.638	2.638
P3	2.654	2.651	2.646	2.645					
P4	3.156	3.144	3.134	3.128	3.124	3.114	3.117	3.115	3.115
P5	4.240	4.203	4.181	4.159	4.148	4.128	4.133	4.129	4.107

TABLE 12 Relative peak areas of principal peaks

Peak areas %									
Peak	101	501	502	503	1101	1102	1103	201	301
P1	18.3	12.3	13.4	15.7	13.4	13.5	11.4	14.4	16.1
P2	10.6	11.7	9.2	12.4	5.2	5.2	5.1	5.8	4.7
P3	10.4	13.3	11.1	8.8					
P4	37.4	45.4	47.8	38.6	43.8	43.4	47.3	46.8	42.2
P5	13.2	8.8	9.6	15.9	22.8	22.6	22.9	18.5	23.6

After storage at 4℃ for 11 months to 3 years, the samples of cardio myopeptidin of this present invention are analyzed by HPLC according to aforesaid chromatographic conditions. It is shown that the retention times of the test samples of 10 batches are similar to the above result. The discrimination index is the proportion of the relative retention time of principal peaks 1, 4 and 5, therein, the sum of the relative percentage of three peaks area is more than 66%, and the proportion of the relative retention time of principal peaks 1, 4 and 5 is 1:1.61:2.14 ( $\pm 0.1$ ) through calculation.

### EMBODIMENT 2

The main pharmacodynamics study for the cardio myopeptidin of the present invention is conducted. The influence and effect of cardio myopeptidin on myocardial morphological index, physiologic index, biochemical indicators, and myocardial oxygen consumption is studied and observed in vitro and in vivo on the myocardial ischemia and ischemia-reperfusion model. The pharmacodynamics experiment and results are as follows:

### 1. Influence of Cardio Myopeptidin on Damage of Myocardial Ultrastructure Caused by Ischemia-Reperfusion

By reference to literature methods, sublingually administer cardio myopeptidin or the reference drug through intravenous injection after rat's coronary artery LAD is ligated for 5 minutes; loosen the ligature after 10 minutes of myocardial ischemia, and reperfusion for 30 min, and simultaneously record II-lead ECG. Blood is taken from the abdominal aorta after the completion of reperfusion, and the heart is perfused and fixed with 6% glutaraldehyde and 0.1 M sodium cacodylate buffer for 2 hours after it is perfused and cleaned with physiological saline water through the aorta. Then the ischemic cardiac muscle from the left frontal wall is picked and cut into 1 mm.sup.3 slices. The slices are immersed in 4% glutaraldehyde and 0.1 M sodium cacodylate buffer to be fixed for the preparation of electron microscope specimens. Slice the specimen of each cardiac muscle after fixation with osmic acid, serial dehydration with acetone, embedment and polymerization with epoxy resin 618, and for each cardiac muscle, cut 4 embedded pieces. Randomly take 20 photos for each group of cardiac muscle of animals with the negative magnification equal to 12000. Observe the change in the ultrastructure, and classify and determine the value according to categories of pathologic changes and severity of damage of mitochondrion, myocardial fiber and other components. The experiments are divided into seven groups, respectively pseudo-operation (P-O) control group and three dose groups such as ischemia-reperfusion (I-R) group, ischemia-reperfusion+normal saline group or +propranolol (I-R+N.S,I-R+Pro) group and ischemia-reperfusion+cardio myopeptidin (I-R+MTP) of three differen dosage group.

TABLE 13 Influence of cardio myopeptidin on semi-quantitative histological assay of cardiac muscle of ischemia-reperfusion in rats observed under electron microscope (n = 20, x  $\pm$  s)

Group	Dosage (mg/Kg)	Value of pathological changes $\pm$ SD
Pseudo-operation control	—	0.36 $\pm$ 0.46
Ischemia-reperfusion	—	1.97 $\pm$ 1.4 $\triangle\triangle\triangle$
Ischemia- reperfusion + normal saline	—	2.68 $\pm$ 1.3*
Ischemia-reperfusion + cardio myopeptidin	1.0	1.85 $\pm$ 1.6*
	5.0	0.73 $\pm$ 0.96***
	10.0	0.33 $\pm$ 0.42***
Ischemia-reperfusion + propranolol	2.0	0.71 $\pm$ 0.84***

Note:Compared with pseudo-operation control group, P < 0.01; compared with ischemia-reperfusion group, \*P > 0.05, \*\*\*P < 0.01.

It is indicated from the experiments that cardio myopeptidin can obviously lessen the damage of myocardial ultrastructure caused by myocardial ischemia-reperfusion, and make or repair it to approach or return to normal condition (as shown in FIG. 6-12).

### 2. Influence of Cardio Myopeptidin on Myocardial Ischemia

Refer to the literature method and make certain modification as required. Lay open the pericardium at LAD after cats are incised with the heart exposed. Render acute myocardial ischemia for 10 minutes through compression method with plastic casing, then loosen for 30 minutes. Sew up the cloth containing five groups (each group has three) of electrodes on the pericardium of the ischemic cardiac muscle. Record I, II

and III-lead electrocardiograms of each group. Simultaneously record the aortic pressure by femoral arterial cannulas. Take ischemia 1', 4' and 7' and reperfuse 1', 5', 10' and 20' and persistently block 1', 5', 10', 15', 20', 30', 40', 50' and 60' as the time of recording. Take the ST elevation and descent expressed in millivolt to represent the change. Block each cat for 5 times, administer different drugs through intravenous injection five minutes before the fourth block, block the LAD persistently for the fifth time, and administer cardio myopeptidin with different doses through intravenous injection in 20 minutes, 30 minutes and 40 minutes after the block, and administer propranolol in 30 minutes after the completion of persistent block. Respectively record and .SIGMA..quadrature.ST and .SIGMA.NST of each group at the third, fourth and fifth block. The experiment cats are divided into 6 groups of: ischemia-reperfusion group (I-R); ischemia-reperfusion in combination with normal saline group (I-R+N.S); ischemia-reperfusion in combination with 2.0, 5.0 or 10.0 mg/kg of cardio myopeptidin groups (I-R+MTP 2.0, 5.0 or 10.0 mg/kg) and ischemia-reperfusion in combination with 2.0 mg/kg of propranolol group (I-R+Pro).

TABLE 14 Effect of prophylactic administration of cardio myopeptidin on .SIGMA..DELTA.ST and .SIGMA.NST at ischemia stage of epicardium electrocardiogram of cats ( $\bar{x} \pm s$ )

Group	Dosage (mg/Kg)	n	$\Sigma \Delta ST$	$\Sigma NST$
Ischemia-reperfusion	—	10	$109 \pm 32$	$28.9 \pm 5.2$
Ischemia-reperfusion + normal saline	—	10	$115 \pm 24^*$	$31.1 \pm 5.1^*$
Ischemia-reperfusion + cardio myopeptidin	2.0	6	$70.8 \pm 16^{***}$	$19.5 \pm 4.8^{***}$
	5.0	6	$37.8 \pm 12^{***}$	$9.33 \pm 3.9^{***}$

Note: Compared with ischemia-reperfusion group,  $*P > 0.05$ ,  $***P < 0.01$

TABLE 15 Effect of therapeutic administration of cardio myopeptidin on .SIGMA..DELTA.ST and .SIGMA.NST of epicardium electrocardiogram of cats ( $\bar{x} \pm s$ )

Group	Dosage (mg/Kg)	n	$\Sigma \Delta ST$	$\Sigma NST$
Ischemia	—	12	$40.5 \pm 10$	$11.1 \pm 2.1$
Ischemia + normal saline	—	12	$40.0 \pm 12^*$	$11.2 \pm 1.5^*$
Ischemia + cardio myopeptidin	2.0	6	$29.9 \pm 2.9^{***}$	$8.67 \pm 2.2^{**}$
	5.0	6	$25.6 \pm 5.7^{***}$	$7.33 \pm 1.5^{***}$
	10.0	6	$19.7 \pm 4.0^{***}$	$6.17 \pm 1.2^{***}$
Ischemia + propranolol	2.0	6	$22.8 \pm 6.4^{***}$	$6.17 \pm 1.5^{***}$

Note: Compared with ischemia group,  $*P > 0.05$ ,  $**P < 0.05$ ,  $***P < 0.01$

The electrocardiogram of the epicardium shows that cardio myopeptidin can obviously antagonize ST elevation caused by myocardial ischemia in cats and reduce the scope of myocardial ischemia.

3. Effect of Cardio Myopeptidin on Release of Myocardial Creatine Phosphokinase (CPK) Caused by Myocardial Ischemia-Reperfusion, Activity of Lactate Dehydrogenase (LDH) and Contents of Free Fatty Acid (FFA) and Malon Dialdehyde (MDA)

(1) Refer to literature methods to make myocardial ischemia-reperfusion damage animal model. Ligate the left anterior descending branch of the coronary artery of rats

for 10 minutes after MTP or verapamil (Ver) is sublingually administered through intravenous injection for 5 min, perfuse for 30 min. Continuously observe II-lead ECG through a polygraph. Take 2 ml left heart blood after the completion of reperfusion, and take the cardiac muscle at the cardiac apex of the left ventricle after the heart is perfused through the aorta. Store the cardiac muscle at 4.°C. and detect within 48 h. Grouping of the experiment: In the experiment, the rats are divided into seven groups: pseudo-operation control group (P); ischemia-reperfusion group (ischemia-reperfusion, I-R); ischemia-reperfusion+normal saline group (I-R+N.S); ischemia-reperfusion +0.5, 2.0 or 10.0 mg/Kg cardio myopeptidin groups (I-R+MTP) and ischemia-reperfusion +1.0 mg/Kg verapamil group (I-R+Ver), 8-10 animals in each group.

TABLE 16 Effect of prophylactic administration of cardio myopeptidin on activities of cardiac muscle and plasma CPK of rats with ischemia-reperfusion in rats ( $\bar{x} \pm s$ )

Group	Dosage (mg/Kg)	n	Cardiac muscle CPK u/100 mg pro	Plasma CPK u/100 ml
Pseudo-operation control		10	980±63	164±64
Ischemia + reperfusion		10	522±65	374±54
Ischemia-reperfusion + normal saline		8	501±59*	337±48*
Ischemia-reperfusion + cardio myopeptidin	0.5	8	732±98***	210±50***
	2.0	8	904±95***	157±31***
	10.0	8	976±95***	134±24***
Ischemia-reperfusion + verapamil	1.0	8	886±115***	192±60***
Ischemia-reperfusion + GMGSP	5.0	8	890±97***	199±35***

Note: Compared with pseudo-operation control group,  $\Delta\Delta\Delta P < 0.01$ ; compared with ischemia-reperfusion group, \* $P > 0.05$ , \*\*\* $P < 0.01$ .

TABLE 17 Effect of prophylactic administration of cardio myopeptidin on activities of cardiac muscle and plasma LDH of rats with ischemia-reperfusion ( $\bar{x} \pm s$ )

Group	Dosage (mg/Kg)	n	Cardiac muscle LDH u/mg pro	Plasma LDH u/ml
Pseudo-operation control	—	10	76.7±19	40.9±9.5
Ischemia + reperfusion	—	10	110±27	120±20
Ischemia-reperfusion + normal saline	—	8	112±19*	116.2±12*
Ischemia-reperfusion + cardio myopeptidin	0.5	8	97.1±12*	93.9±17***
	2.0	8	76.3±22***	59.7±12***
	10.0	8	64.8±17***	52.6±13***
Ischemia-reperfusion + verapamil	1.0	8	75.1±23***	46.7±8.8***
Ischemia-reperfusion + GMGSP	5.0	8	83.0±17***	60.9±15***

Note: Compared with pseudo-operation control group,  $\Delta\Delta\Delta P < 0.01$ ; compared with ischemia-reperfusion group, \* $P > 0.05$ , \*\*\* $P < 0.01$ .

TABLE 18 Effect of prophylactic administration of cardio myopeptidin on cardiac muscle and plasma MDA content of rats with ischemia-reperfusion ( $\bar{x} \pm s$ )

Group	Dosage (mg/Kg)	n	Cardiac muscle MDA nmol/100 mg pro	Plasma MDA nmol/ ml
Pseudo-operation control		10	68.3±8.4	22.3±1.8
Ischemia + reperfusion		10	135±10	63.6±11
Ischemia-reperfusion + normal saline		8	127±15*	58.4±11*
Ischemia-reperfusion + cardio myopeptidin	0.5	8	73.1±13***	38.1±6.2***
	2.0	8	60.5±10.4***	27.7±5.5***
	10.0	8	49.8±9.4***	25.5±5.1***
Ischemia-reperfusion + verapamil	1.0	8	66.6±19.8***	24.9±6.6***
Ischemia-reperfusion + GMGSP	5.0	8	75.2±9.7***	39.2±5.3***

Note: Compared with pseudo-operation control group,  $\triangle\triangle\triangle P < 0.01$ ; compared with ischemia-reperfusion group, \* $P > 0.05$ , \*\*\* $P < 0.01$ .

TABLE 19 Effect of cardio myopeptidin on plasma FFA content of rats with ischemia-reperfusion (n = 8,  $\bar{x} \pm s$ )

Group	Dosage (mg/Kg)	FFA mol/100ml
Pseudo-operation control	—	60.6±7.8
Ischemia + reperfusion	—	129±26
Ischemia-reperfusion + normal saline	—	121±10*
Ischemia-reperfusion + cardio myopeptidin	1.0	85.4±5.0***
	5.0	77.7±7.1***
	10.0	71.4±11***
Ischemia-reperfusion + propranolol	2.0	77.1±6.4***
Ischemia-reperfusion + GMGSP	5.0	89.2±6.7***

Note: Compared with pseudo-operation control group,  $\triangle\triangle\triangle P < 0.01$ ; compared with ischemia-reperfusion group, \* $P > 0.05$ , \*\*\* $P < 0.01$ .

Compared with the growth-stimulating peptide of the myocardial cells (GMGSP) disclosed in Chinese patents of ZL94102798 and ZL94102799, cardio myopeptidin of the present invention obviously has higher in vitro biological activity. The biological activity of said cardio myopeptidin is from 3 to 5 times higher than that of the growth-stimulating peptide of the myocardial cells. Comparison data of in vivo results show it poses a favorable impact on the release of myocardial creatine phosphokinase, biological activity of lactate dehydrogenase, and contents of free fatty acid and malon dialdehyde caused by myocardial ischemia-reperfusion injury (as shown in Table 16-19).

(2) Refer to literature methods to make Langendorff's cardiac hypoxia-reoxygenation damage animal model of isolated rat heart. Conduct Langendorff's perfusion with K--H liquid with high  $Ca^{2+}$  and low  $K^{+}$  and persistent filling of mixed gas. Hook two platinum filaments on the apex of heart and the root of the left cardiac atrium respectively, and record the electrocardiogram. Ligate LAD for 10 min and loosen for 15 min. Conduct perfusion with K--H liquid with corresponding concentrations of cardio myopeptidin from 5 min before ligation to 5 min after loosening for the cardio myopeptidin-treated group. Determine the related indexes of effluent before and after 8 min of ligation and 2 min after loosening



respectively. Take the cardiac muscles of the frontal and posterior wall of the left ventricle after the completion of perfusion. Store the muscles at 4.°C. and detect their CPK, LDH and MDA within 48 h. In the experiment, rats are divided into 6 groups: pseudo-operation control group (P-O), hypoxia-reoxygenation group (anoxia-reoxygenation, A-R), hypoxia-reoxygenation+10, 50 or 100 .mu.g/ml cardio myopeptidin (final concentration, A-R+MTP) groups and hypoxia-reoxygenation+1.0 .mu.g/Kg verapamil (A-R+Ver) group, and each group contains 10 animals.

TABLE 20 Effect of cardio myopeptidin on activity of CPK of coronary effluent of isolated rats with myocardial ischemia-reperfusion (n = 10, x  $\pm$  s)

Group	Dosage ( $\mu$ g/ml)	Coronary effluent CPK (U/L)		
		Prior ischemia	During ischemia	Perfusion
Pseudo-operation control	—	15.3 $\pm$ 1.5	16.5 $\pm$ 1.8	17.1 $\pm$ 2.0
Ischemia + reperfusion	—	16.3 $\pm$ 2.3 $\Delta$	24.8 $\pm$ 2.7 $\Delta\Delta\Delta$	35.4 $\pm$ 4.3 $\Delta\Delta\Delta$
Ischemia-reperfusion + cardio myopeptidin	10	16.1 $\pm$ 2.6*	20.7 $\pm$ 1.7***	22.7 $\pm$ 2.3***
	50	15.6 $\pm$ 1.7*	17.9 $\pm$ 2.7***	19.0 $\pm$ 2.3***
	100	15.5 $\pm$ 2.7*	15.3 $\pm$ 2.1***	16.5 $\pm$ 2.4***
Ischemia-reperfusion + verapamil	1	16.3 $\pm$ 2.0*	16.2 $\pm$ 2.8***	16.0 $\pm$ 1.8***

Note: Compared with pseudo-operation control group,  $\Delta P > 0.05$ ,  $\Delta\Delta\Delta P < 0.01$ ; compared with ischemia-reperfusion group, \* $P > 0.05$ , \*\*\* $P < 0.01$

TABLE 21 Effect of cardio myopeptidin on activity of LDH of coronary effluent of isolated rats with myocardial ischemia-reperfusion (n = 10, x  $\pm$  s)

Group	Dosage ( $\mu$ g/ml)	Coronary effluent LDH (U/L)		
		Prior ischemia	During ischemia	Perfusion
Pseudo-operation control	—	11.8 $\pm$ 0.79	12.6 $\pm$ 1.1	11.8 $\pm$ 0.69
Ischemia + reperfusion	—	11.7 $\pm$ 0.83 $\Delta$	17.3 $\pm$ 1.9 $\Delta\Delta\Delta$	24.7 $\pm$ 1.7 $\Delta\Delta\Delta$
Ischemia-reperfusion + cardio myopeptidin	10	12.0 $\pm$ 0.58*	13.4 $\pm$ 1.1***	15.3 $\pm$ 1.4***
	50	11.8 $\pm$ 0.53*	12.9 $\pm$ 1.1***	13.4 $\pm$ 0.76***
	100	11.2 $\pm$ 0.55*	12.2 $\pm$ 0.79***	12.9 $\pm$ 0.93***
Ischemia-reperfusion + verapamil	1	11.4 $\pm$ 0.78*	13.0 $\pm$ 0.62***	14.3 $\pm$ 0.95***

Note: Compared with pseudo-operation control group,  $\Delta P > 0.05$ ,  $\Delta\Delta\Delta P < 0.01$ ; compared with ischemia-reperfusion group, \* $P > 0.05$ , \*\*\* $P < 0.01$

Experiments indicated that cardio myopeptidin could obviously decrease the release of myocardial creatine phosphokinase, and the increase of activity of lactate dehydrogenase and contents of free fatty acid and malon dialhedyde caused by myocardial ischemia-reperfusion.

#### 4. Effect of Cardio Myopeptidin on Myocardial Oxygen Consumption

Anesthetize dogs with sodium pentobarbital. Conduct endotracheal intubation and artificial respiration, and use an RM-86 polygraph to monitor electrocardiogram and aortic pressure. Open the thoracic cavity from the left side to expose the heart. Intubate a cannula from the apex of the heart to the left ventricle, and record the pressure of the left ventricle and pressure change rate ( $\pm dp/dt$  max). In order to know the change in circulation of the coronary artery and myocardial oxygen metabolism, separate the left circumflex branch of the coronary artery of dogs, use an

electromagnetic flowmeter to measure the flow of coronary artery, and calculate the resistance against the coronary artery. Intubate a cannula from the external jugular vein of dogs to the coronary artery. Simultaneously draw the arterial blood and the coronary sinus blood. Use a blood gas analyzer (Model ABL-3, Denmark) to determine blood oxygen content, and calculate myocardial oxygen uptake and myocardial oxygen consumption. Keep the arterial blood pH, CO<sub>2</sub> and partial pressure of oxygen of dogs within normal range. The dose of MTP is 2.5 and 10 mg/kg, and the interval between two doses is 30 min. Continuously record various parameters after administration until they return to the control value. Take arterial blood and coronary sinus blood at 2, 5, 10 and 30 minutes after administration. Determine blood gas content, calculate myocardial oxygen uptake and myocardial oxygen consumption, and observe the effect of MTP on myocardial oxygen metabolism.

TABLE 22 Effect of cardio myopeptidin for intravenous injection on myocardial oxygen consumption and myocardial oxygen uptake in dogs (change value % after administration)

Time(min)	myocardial oxygen consumption (MVO <sub>2</sub> )	myocardial oxygen uptake (O <sub>2</sub> ext)
Cardio myopeptidin 2 mg/kg (N=8)		
2	-23.0±26	-4.00±13
5	-21.0±13***	0±8.0
10	18.0±14	-2.00±6.0
20	-9.00±12	-2.00±12
Cardio myopeptidin 5 mg/kg (N=7)		
2	-36.0±24**	-5.00±13
5	-26.0±21**	6.00±8.0
10	-19.0±15**	6.00±6.0*
20	-8.00±10	-14.0±35
Cardio myopeptidin 10 mg/kg (N=6)		
5	-22.0±25	9.00±5***
10	-21.0±14**	2.00±5.0
20	-8.00±4.0*	3.00±4.0
30	-10.0±7.0*	-6.00±15
Propranolol 2 mg/kg (N=6)		
2	-31.0±13*	-3.00±1.0
5	-30.0±13***	3.00±7.0
10	-33.0±10***	3.00±8.0
30	-32.0±14***	3.00±9.0

Note: Compared with that before administration: \*P > 0.05, \*\*P < 0.05, \*\*\*P < 0.01

#### 5. Effect of Cardio Myopeptidin on Myocardial Infarction

Anesthetize a healthy, grown male miniature pig with the body weight of 20.9±4.0 kg with 30 mg/kg of 3% sodium pentobarbital through auditory intravenous injection.

Connect the tracheal cannula to a SC-3 electro-respirator to perform artificial positive pressure respiration. Open the thoracic cavity from the third rib at the left side to expose the heart. Separate the anterior descending branch of the coronary artery (about 1/3 distant from the apex of heart), and put a silk thread 0# beneath it for ligation. Place a multi-point fixed type epicardium electrode with 20 points on the myocardial surface under the ligature. Record the myocardial electrical signals on an RTA-1200 model hot-wave recorder of a RM-6300 model eight-lead polygraph through a ZYS1-I model numerical control epicardium scanner and AB-601G bioelectric amplifier, and 1 mV standard voltage is equal to 1 mm. Under the control of the automatic timer, measure the change in the electrocardiogram at those 20 points. Intubate a cannula from the femoral artery to the abdominal aorta, and connect it to a AP-641G blood pressure amplifier through a TP-400T model pressotransducer to measure the mean blood pressure (MBP). Insert a needle electrode into the subcutaneous tissue of the four limbs, and use a AC-601G electrocardiogram amplifier to measure standard II-lead electrocardiogram (ECG.quadrature.), and input the electrical signal of the ECG into an AT-601G cardiograph to measure the heart rate (HR). A femoral venous cannula is used for administration and fluid replacement.

In the experiment, the animals are divided into four groups and a total of 25 animals are experimented upon. When infusing 120 mg/kg of mannitol through phleboclysis for the control group, 5 animals die due to ventricular fibrillation, and 5 animals survive, so each of the other groups has five animals. They are respectively infused with 5 and 10 mg/kg experiment drug and 0.25 mg/kg positive drug verapamil through the femoral vein respectively. The dosage is 2 ml/kg, and the infusion rate is 2 ml/min. Trace the electrocardiogram after various indexes stabilize upon the completion of the operation. Ligate the anterior descending branch, then record the electrocardiogram after 5 min as the control before administration. Then administer the drug through phleboclysis. Record ECG II, MBR, HR and ST elevation values of the electrocardiogram at those 20 points respectively at 5, 10, 15, 20, 25, 30, 45, 60, 90, 120 and 180 min after the administration. Calculate the sum ST and take it as the index to measure the degree of myocardial ischemia. Set the point at which ST elevation of electrocardiogram exceeds 2 mV as the ischemic point, and calculate the total ischemic points (NST) as the index to measure the range of myocardial ischemia. At 3 h after the administration, bleed the animals to execute them and rapidly take out the heart. Cut out the ventricles and wash away the residual blood, and slice the ventricles under the location of ligature into 5 mm thick coronal-shape pieces. Keep the pieces away from light and dye them with 1% TTC at room temperature for 30 min. Then mark the ischemic region and non-ischemic region of both sides of 5 cardiac muscles on transparent film. Cut the film of the white infarct region and weigh. Use this weight to divide the weight of film of 10 sides of the ventricles, and use the result to calculate the percentage of infarct region in ventricular weight under the ligature.

Grouping, dosage and administration pattern of the experiment

Group	Drug	Dose infused (mg/kg)	Infusion rate (ml/min)
Control group with solvent administered	Mannitol	120	2
Low-dose group of tested drug	Cardio myopeptidin + mannito	5+120	2

High-dose group of tested drug	Cardio myopeptidin + manntiol	10+120	2
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Control group with positive drugs administered	Verapamil	0.25	2
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TABLE 23 Effect of cardio myopeptidin for phleboclysis on scope of myocardial infarction in pigs

Drug	Dose (mg/kg)	Animal number(n)	Infarction range(%)
solvent control group	-	5	19.4±3.02
cardio myopeptidin	5	5	11.8±3.13*
cardio myopeptidin	10	5	10.2±3.2**
Verapamil	0.25	5	12.5±3.4*

Note: Compared with solvent control group, \*P < 0.05, \*\*P < 0.01.

It is demonstrated from the experiments that 5 and 10 mg/kg of cardio myopeptidin of the present invention can obviously lower ST of the electrocardiogram of pigs with myocardial infarction, decrease NST and reduce the scope of myocardial infarction. Cardio myopeptidin of the present invention has certain therapeutic action on arrhythmia and ventricular fibrillation (they may cause death) in pigs with acute myocardial ischemia, but poses no evident impact on blood pressure and heart rate.

### EMBODIMENT 3

1 kg of ventricular myocardium of healthy infant pigs is cleaned and minced; 1 kg of sterile distilled water is added to the minced ventricular myocardium to homogenize under the rotation speed of 3000 rpm/min. The homogenate is frozen at -20.°C. for 24 h subsequently melted, then the homogenat is heated to 75.°C. in a water bath after the homogenate is frozen and thawed, repeating 3 times. The heated homogenate is filtered with a XAS03-172/8 plate-and-frame filter (purchased from Guangzhou Medicinal Apparatus Research Institute) with pores of 10.mu. of medium-speed filter paper to obtain the coarse filtrate, and the residue is discarded. The coarse filtrate is ultrafiltered by a hollow-fiber column (specification of F60, purchased from Sweden Gambro Corporation) to obtain the fine filtrate with the molecular weight of 12 Kd. The fine filtrate is ultrafiltered by ultrafiltration membrane (10 Kd, Millipore Corporation) wherein 150 ml of cardio myopeptidin solution with the molecular weight of 9500 Da is intercepted. The obtained cardio myopeptidin solution is concentrated with a reverse osmosis and concentration column provided by Millipore Corporation.

The cardio myopeptidin solution is inspected for quality until it meets the quality standard, then a aseptic filtration, filling and lyophilization (lyophilizer is used) is performed respectively. The procedure of lyophilization comprises the steps of: the shelf in the drying chamber is cooled down to -20°C in 20 minutes. The cardio myopeptidin solution is then frozen to -35°C in 30 minutes and stands for 2 hours in such condition. The temperature within the condenser is chilled to -50.°C., then the pressure is reduced until the vacuum degree reaches 100 Kpa. The drying chamber is connected with the condenser, and the refrigeration of the drying chamber is stopped, when vacuum degree of the drying chamber reaches 15 Pa, the temperature in the drying chamber is increased to 15°C at the rate of 3.°C/min and kept for 3 hours, and the temperature is raised continuously to 22.°C. at the rate of 10°C/min and maintained for 5 hours. Then the temperature is raised continuously to 35°C at the rate of 10.°C./min and kept for 2 hours, whereafter the temperature is raised to 5°C at

the rate of 5.°C/min for 1 h. Then in the cooling stage, the temperature is reduced to 40.°C. within 20 min and maintained for 10 hours. Thus, lyophilized cardio myopeptidin with qualified appearance is obtained, and the product is taken out for sealing.

It is shown by analysis that the polypeptide content of the obtained cardio myopeptidin is 85%, free amino acid content is 8%, ribonucleic acid content is 1%, deoxyribonucleic acid content is 6%, and the average molecular weight is 9500 Da.

#### **EMBODIMENT 4**

1 kg of ventricular myocardium from healthy infant cattle is cleaned and minced, and 1 kg of sterile distilled water is added to the minced ventricular myocardium to homogenize under the rotation speed of 5000 rpm/min. The homogenate is frozen at -30.°C. for 48 h subsequently melted, then the homogenate is heated to 90.°C. at a water bath after the homogenate is frozen and thawed, repeating 4 times. The heated homogenate is filtered with a XAS03-172/8 plate-and-frame filter (purchased from Guangzhou Medicinal Apparatus Research Institute) with pores of 8.μm. of medium-speed filter paper to obtain the coarse filtrate, and the residue is discarded. The coarse filtrate is ultrafiltered by a hollow-fiber column (specification of F60, purchased from Sweden Gambro Corporation) to obtain the fine filtrate with the molecular weight of 12 Kd. The fine filtrate is ultrafiltered by ultrafiltration membrane (5 Kd, Millipore Corporation) wherein 150 ml of cardio myopeptidin solution with the molecular weight of 5000 Da is intercepted. The obtained cardio myopeptidin solution is concentrated with a reverse osmosis and concentration column provided by Millipore Corporation.

The cardio myopeptidin solution is inspected for quality until it meets the quality standard, then an aseptic filtration, filling and lyophilization (the equipment used is a lyophilizer) is performed respectively. The procedure of lyophilization comprises the steps of: the shelf in the drying chamber is cooled down to -18°C in 40 minutes. The cardio myopeptidin is then frozen to -25°C in 20 minutes, maintaining at this temperature for 1 hour. Then the condenser is chilled to -40°C, then the pressure is reduced until the vacuum degree reaches 95 Kpa. The drying chamber is connected with the condenser, and the refrigeration of the drying chamber is stopped, when the vacuum degree of the drying chamber reaches 12 Pa, the temperature of the drying chamber is raised to 10°C at the rate of 2.°C/min and maintained for 5 hours. The temperature is raised continuously to 25°C at the rate of 16.°C/min and maintained for 5 hours. Then the temperature is raised continuously to 35°C at the rate of 10.°C/min and maintained for 3 hours, whereafter the temperature is raised to 60°C at the rate of 8.°C/min and maintained for 2 h. Then in the cooling stage, the temperature is reduced to 46.°C. within 30 min and maintained for 8 hours. Thus, lyophilized cardio myopeptidin with qualified appearance is obtained, and the product is taken out for sealing.

Through analysis for cardio myopeptidin obtained in this embodiment, its polypeptide content is 78%, free amino acid content is 15%, ribonucleic acid content is 2%, deoxyribonucleic acid content is 5%, and the average molecular weight is 5000 Da.

#### **EMBODIMENT 5**

1 kg of ventricular myocardium from healthy infant rabbits is cleaned and minced, and 1 kg of sterile distilled water is added to the minced ventricular myocardium to homogenize under the rotation speed of 1000 rpm/min. The homogenate is frozen at

-10.°C. for 72 h and subsequently melted, then the homogenate is heated to 85.°C. in a water bath after the homogenate is frozen and thawed, repeating 3 times. The heated homogenate is filtered with a XAS03-172/8 plate-and-frame filter (purchased from Guangzhou Medicinal Apparatus Research Institute) with pores of 5  $\mu$  of medium-speed filter paper to obtain the coarse filtrate, and the residue is discarded. The coarse filtrate is ultrafiltered by a hollow-fiber column (specification of F60, purchased from Sweden Gambro Corporation) to obtain the fine filtrate with the molecular weight of 11 Kd. The fine filtrate is ultrafiltered by ultrafiltration membrane (3 Kd, Millipore Corporation) wherein 150 ml of cardio myopeptidin solution with the molecular weight of 2000 Da is intercepted. The obtained cardio myopeptidin solution is concentrated with a reverse osmosis and concentration column provided by Millipore Corporation.

The quality inspection is performed with the cardio myopeptidin solution until it meet the quality standard, then a aseptic filtration, filling and lyophilization (the equipment used is a lyophilizer) is performed respectively. The procedure of lyophilization comprises the step of: the shelf in the drying chamber is cooled down to -15.°C. in 10 minutes, then the cardio myopeptidin solution is cooled down to -30.°C. in 25 minutes and the temperature is maintained for 2.5 hours. The temperature within the condenser is cooled down to -45.°C., then the pressure is reduced until the vacuum degree reaches 90 Kpa. The drying chamber is connected with the condenser, and the refrigeration of the drying chamber is stopped, when the vacuum degree of the drying chamber reaches 10 Pa, the temperature in the drying chamber is raised to 5.°C. at the rate of 5°C/min and maintained for 6 hours, and the temperature is raised continuously to 15.°C. at the rate of 8°C/min and maintained for 8 hours, then the temperature is raised continuously to 32.°C. at the rate of 7°C/min and maintained for 4 hours, whereafter the temperature is raised to 55°C at the rate of 4°C/min and stayed for 3 h. Then in the cooling stage, the temperature is reduced to 50.°C. within 10 min. and maintained at such temperature for 15 hours. Thus, lyophilized cardio myopeptidin with qualified appearance is obtained, and the product is taken out for sealing.

It is shown by analysis that the polypeptide content of obtained cardio myopeptidin is 90%, free amino acid content is 6%, ribonucleic acid content is 1%, deoxyribonucleic acid content is 3%, and average molecular weight is 2000 Da.

#### **EMBODIMENT 6**

Identical with Embodiment 3, the difference is that cardio myopeptidin solution with intercepted molecular weight of 4000 Da is tested up to the quality standard through quality inspection, then subjected to aseptic filtration and filling, and is prepared according to the following procedure:

cardio myopeptidin	20 mg
mannitol	375 mg
activated carbon	0.005 mg
water for injection	Add to 5 ml

Fill the solution into bottles and place them in a lyophilizer. Cool down the shelf in the drying chamber to -20.°C. in 30 minutes, then after 40 minutes cool down the product to -35.°C. and maintain at such temperature for 3 hours. Cool down the temperature within the condenser to -50.°C., then reduce the pressure. Connect the drying chamber and the condenser when the vacuum degree reaches 95 Kpa, and stop the refrigeration of the drying cabinet, begin to raise the temperature to 10°C at the rate of 3.°C./min, and incubate for 4 hours when vacuum degree of the drying cabinet

is 15 Pa. Continue to raise the temperature to 20.°C. at the rate of 12°C/min, maintained for 5.5 hours. Continue to raise the temperature to 30°C at the rate of 12. °C./min, maintain for 1.5 h. Continuously raise the temperature to 60.°C. at the rate of 6°C/min, maintain for 2 h. Then in the cooling stage, cool down the temperature to 48. °C. within 20 min and maintain at such temperature for 9 hours. Thus, obtain the lyophilized cardio myopeptidin product with qualified appearance. Take out the product and seal.

Through lyophilization, the finished product with cardio myopeptidin content of 2.0 mg/ml is obtained. Through analysis for said cardio myopeptidin, its polypeptide content is 80%, free amino acid content is 12%, ribonucleic acid content is 2%, deoxyribonucleic acid content is 6%, and the average molecular weight is 4000 Da.

#### **EMBODIMENT 7**

An essentially identical process is performed according to Embodiment 3. The difference is that the raw material is the ventricular myocardium of healthy infant horses, and the cardio myopeptidin solution with intercepted molecular weight of 8000 Da is obtained. Analysis indicated that the polypeptide content of cardio myopeptidin is 84.5%, free amino acid content is 6%, ribonucleic acid content is 2%, deoxyribonucleic acid content is 7.5%, and average molecular weight is 8000 Da.

#### **EMBODIMENT 8**

An essentially identical process is performed according to Embodiment 3. The difference is that cardio myopeptidin solution further comprises trehalose, and the component ratio is: 15 mg/ml cardio myopeptidin: 200 mg/ml trehalose.

#### **EMBODIMENT 9**

An essentially identical process is performed according to Embodiment 1. The difference is that the raw material is the ventricular myocardium of healthy pigs, the cardio myopeptidin solution further comprises lactose, and the component ratio is: 18 mg/ml cardio myopeptidin: 250 mg/ml lactose.

#### **EMBODIMENT 10**

An essentially identical process is performed according to Embodiment 6. The difference is that cardio myopeptidin solution with intercepted molecular weight of 1000 Da is obtained, and the components of cardio myopeptidin solution are:

cardio myopeptidin	16 mg
sucrose	300 mg
activated carbon	0.005 mg
water for injection	Add to 5 ml

Analysis indicated that the polypeptide content of cardio myopeptidin is 82%, free amino acid content is 12%, ribonucleic acid content is 2%, deoxyribonucleic acid content is 4%, and the average molecular weight is 1000 Da.

#### **EMBODIMENT 11**

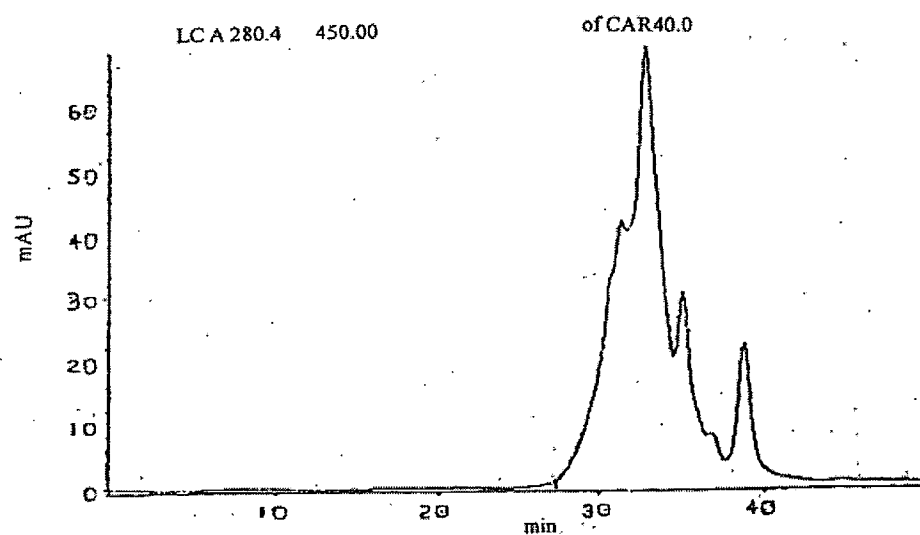
Preliminary stability test on cardio myopeptidin

1. The results of influencing factor test and accelerated test on cardio myopeptidin for injection show, after the external packaging is removed, the appearance rapidly turns yellow under the conditions that humidity is more than 75% and temperature exceeds 37.°C., moisture content increases and activity decreases.

2. The result of room-temperature filed sample inspection shows, except the appearance turns light yellow after from 480 to 540 days, other test items present no change, and various test items have no change if the drug is stored at 4°C. This demonstrates cardio myopeptidin can be stored for 150 days at least at room temperature under the situation in which humidity is from 45% to 90%, and

characteristics of its appearance, content and activity fail to show change, and it can be stored at 4°C for 480 days at least.





LCA 280.1 450.00 of CAR40.0  
DATA GAR40.0

(Peaks)	(Ret Time)	(Type)	(Width)	(Area)	(Start Time)	(End Time)	
1	31.263	UU	1.205	4412	26.896	31.623	5165
2	32.747	UU	1.331	7448	31.623	34.463	3709
3	36.012	UU	0.955	2143	34.463	36.572	2236
4	38.731	UU	0.774	1296	37.714	41.490	975

$$X=6.7447-0.09697X \quad Y=-0.9937$$

FIG.1

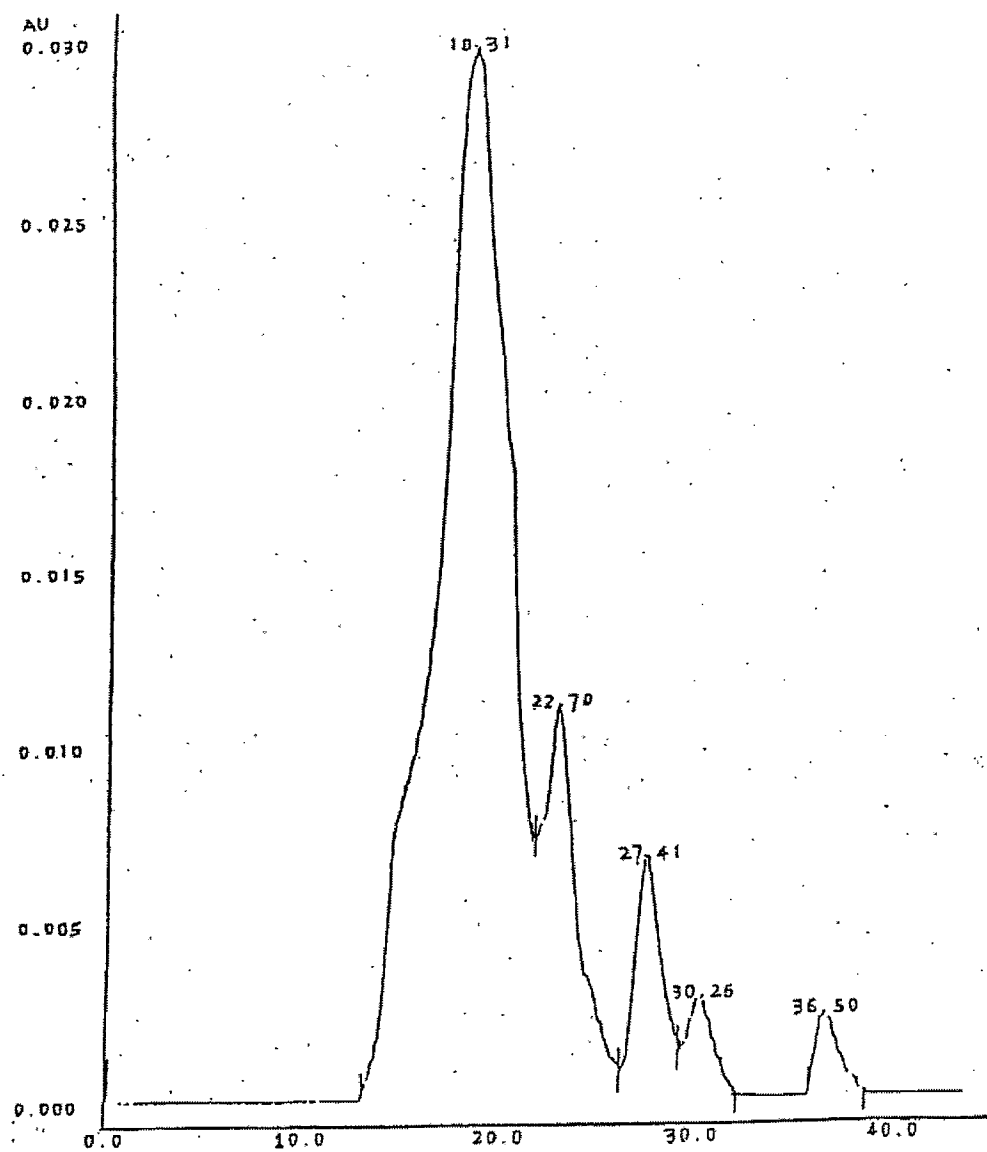


FIG.2

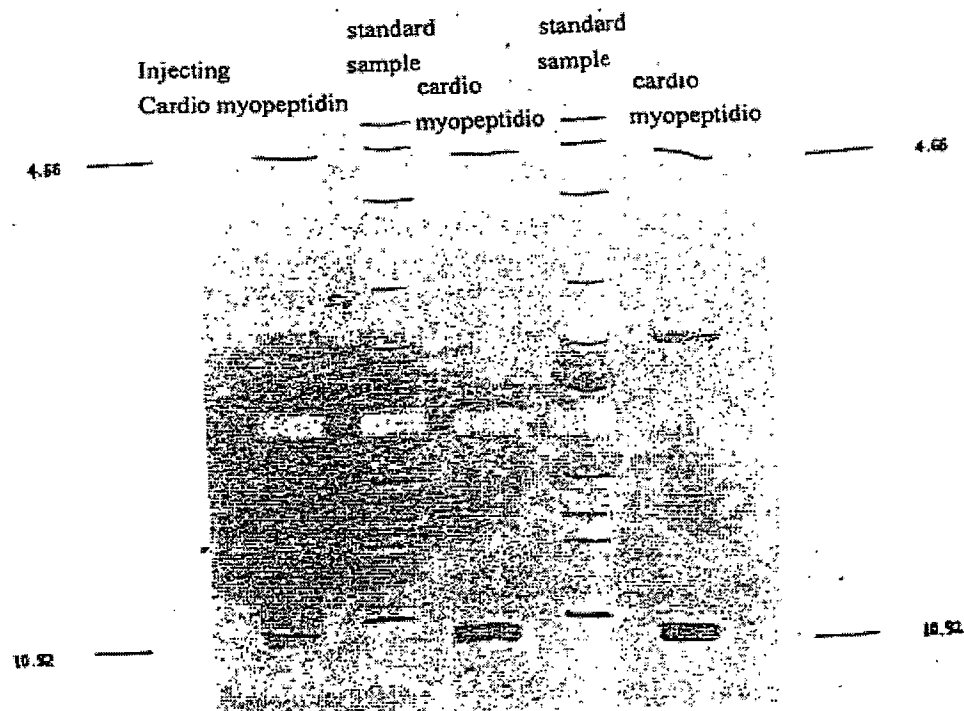
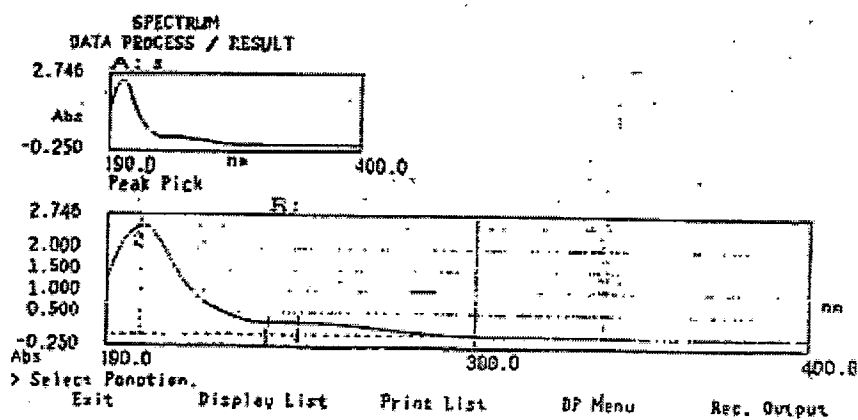
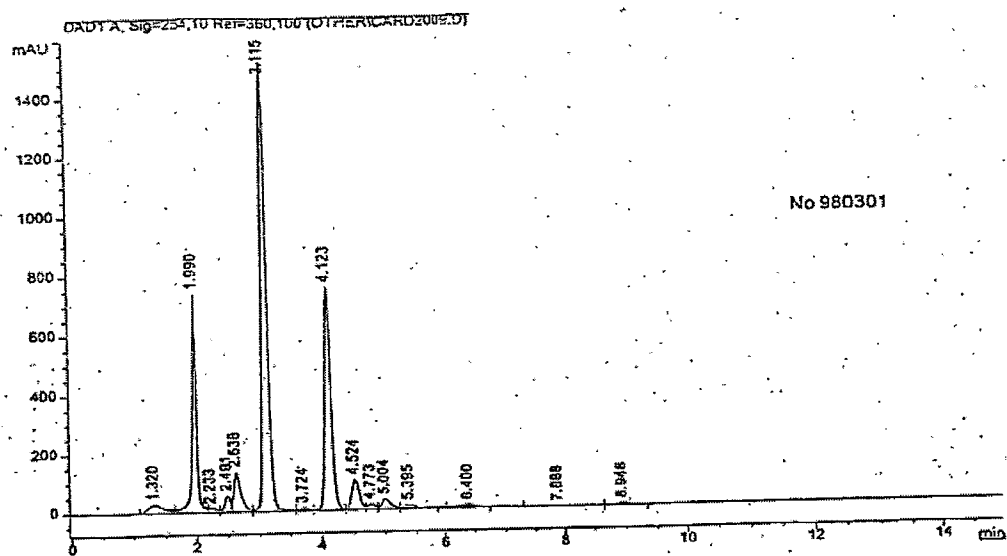


FIG3



NO.	ARSCISSA	PEAK	HEIGHT	ABSCISSA	VALIEY	HEIGHT
1	247.4	0.2884	0.0239	238.8	0.2818	-0.4106
2	200.4	2.4966	1.4000			

FIG4



# Area Percent Report

Sorted By : Signal  
Multiplier : 1.0000  
Dilution : 1.0000

Signal 1: DAD1 A, Sig=254,10 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.320	VV	0.2476	523.23895	28.84586	2.3415
2	1.990	VV	0.0687	3608.51245	742.13055	16.1483
3	2.233	VV	0.1214	127.18691	14.92828	0.5692
4	2.491	VV	0.1013	371.21744	55.99518	1.6612

FIG5



FIG.6



FIG.7



FIG.8



FIG.9



FIG.10



FIG.11

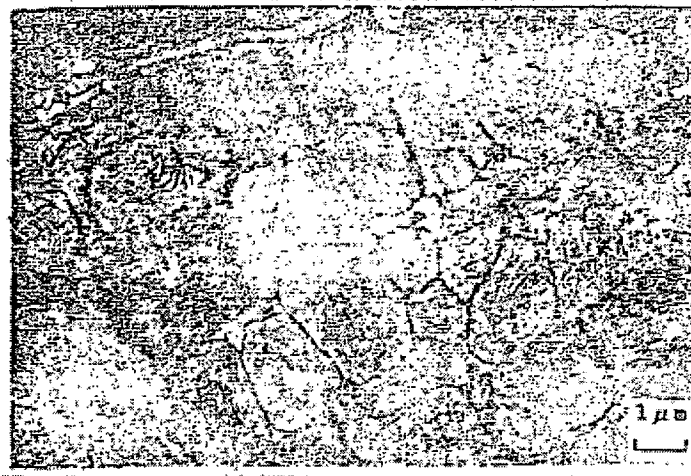


FIG.12

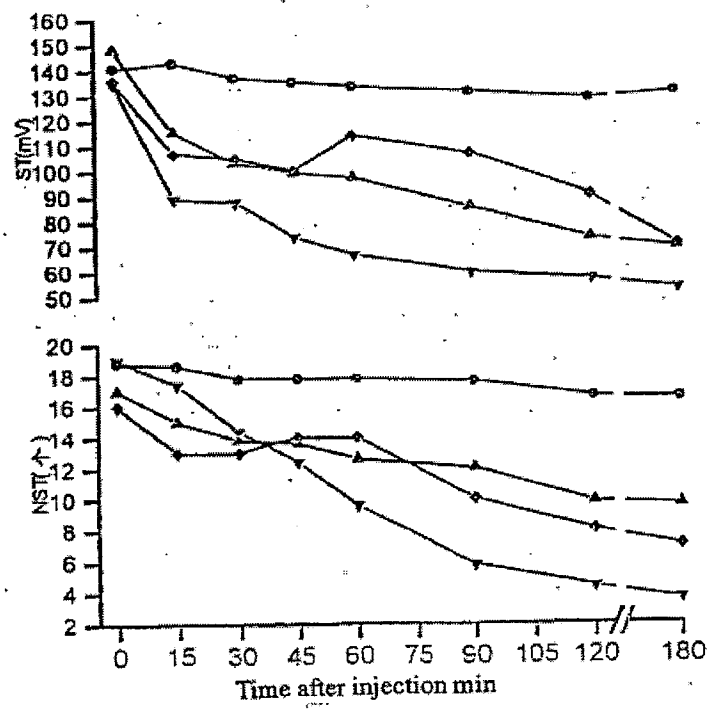


FIG.13